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CRISTISPIRA IN NORTH AMERICAN SHELLFISH. A NOTE ON A SPIRILLUM FOUND IN OYSTERS.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 21 to 24.

(Received for publication, April 1, 1921.)

In the European lamellibranchiata there have been found a large number of comparatively coarse forms of spiral organisms, provided with a broad, band-like membrane extending from one extremity to the other and winding itself obliquely around the body, one edge being attached to the body, the other free. The organism was first discovered in oysters by Certes (1), who regarded it as akin to the trypanosome and called it *Trypanosoma balbianii*. Laveran and Mesnil (2) considered it a bacterium, while Perrin (3), working under Schaudinn, thought it a protozoon. At that time Schaudinn (4) held the view that the spirochete represented a phase of the trypanosome and that both belonged to the protozoa. The peculiar structure of the organism, as brought out by the technique employed by Perrin, suggested the mitotic and other nuclear figures to such an extent that the organism was accepted as a protozoon by certain protozoologists (5–8). The investigations by Swellengrebel (9) and later by Gross (10) led the latter to place it in the class of *Cristispira*, a terminology accepted by Dobell (11) and Zuelzer (12). The points in dispute are: (1) the interpretation of the membrane; (2) the presence or absence of a periplast; and (3) the presence or absence of a nuclear apparatus, especially the significance of the chambered structure of the body. These questions, which had never been definitely settled, led the writer to undertake examinations of American shellfish. Furthermore, so many species have been described recently in the European shellfish that a comparison from the standpoint of geographic distribution of these cristispiras seemed desirable.

TABLE I.

Spirochæta (*Schellack*).

Name.	Source.	Length.		Breadth.		Ends.
		Average.	Extremes.	Average.	Extremes.	
Spirochæta balbianii.	*Ostrea edulis.*	39μ	35–42μ	1.3μ	1.1–1.5μ	Rounded, no terminal appendage.
" *ostreæ.*	" "	41.5μ	38–42.5μ	1.1μ	1.0–1.3μ	Sharp, " " "
" *chamæ.*	*Chama gryphoides.* " *sinistrorsa.*	45.6μ	45–46.5μ	1.4μ	1.3–1.5μ	Rounded, " " "
" *anodontæ.*	*Anodonta mutabilis.*	46μ	39–50.5μ	1.0μ	0.9–1.2μ	" " " "
" *spiculifera.*	"	33μ	28–36.5μ	0.9μ	0.7–1.1μ	Pointed, terminal filament.
Spirochæta modiolæ.	*M. barbata.*	37.5μ	36–40μ	0.8μ	0.7–0.9μ	Rounded, no terminal appendage.
" *pinnæ.*	*P. nobilis.*	30.4μ	29–31μ	1.0μ	0.8–1.1μ	" " " "
" *limæ.*	*L. inflata, L. hians.*	37μ	35–41μ	1.4μ	1.0–1.8μ	" " " "
" *cardii papillos.*	*C. papillosum.*	19.1μ	18.5–20μ	1.2μ	1.1–1.4μ	" " " "
Spirochæta tapetos.	*T. decussata.*	34.5μ	29–35μ	1.3μ	1.1–1.4μ	Rounded, occasionally terminal appendage.
" *acuminata.*	*Tapes læta.*	47μ	43.5–49.5μ	1.0μ	0.9–1.1μ	Pointed, no terminal appendage.
Spirochæta saxicavæ.	*Sax. arctica.*	31μ	30–32μ	1.7μ	1.6–1.8μ	Rounded, " " "
" *gastrochænæ.*	*G. dubia.*	29μ	Constant.	1.2μ	1.1–1.3μ	One end blunt, one sharp, no terminal appendage.
*Spirochæta pusilla.**	*Anodonta, Unio, Lima, Tapes, etc.*	13μ	12–14μ		0.3–0.4μ	Sharp, pointed.

* Bosanquet found a spirochete 10 to 12 μ in length which he thinks may be identical with *Spirochæta hartmanni* of Gonder or with *Spirochæta pusilla* of Schellack. (No crista?)

Schellack (13), Keysselitz (5), and Gross (10, 14) created a large number of species with only slight morphological variations. To quote an example, Schellack set up thirteen species with certain characteristics, as shown in Table I.

As will be pointed out later, the method of classifying these coarse organisms by their morphological features is subject to error because of the great variations in the terminal portions and in the thickness of body according to the degree of fixation, dehydration, or staining; a difference in these factors may cause the same organism to assume a totally different aspect. Hence the writer has studied these organisms not only in stained preparations but also under the dark-field microscope.

TABLE II.

Occurrence of Cristispira in the Styles of North American Shellfish.

Shellfish.	No. of specimens examined.	Styles present.	*Cristispira* present.
Ostrea virginiana	298	128	99 (40 degenerated).
Venus mercenaria	110	70	8 (3 ").
Modiola modiolus	97	73	4
Ensis americana	24	20	0
Mya arenaria	24	24 (hard).	0
Mactra solidissima	12 (dead?).	1	0
Mytilus edulis	42	34	0
Pecten irradians	39	34 (hard).	0
Fulgur canaliculatus	8	2 (?)	0
Nassa obsoleta	30	4	0

The present study deals only with salt water shellfish caught in the neighborhood of Woods Hole during the month of August, 1916, and includes *Ostrea virginiana* (oysters), *Venus mercenaria* (quahaugs), *Mya arenaria* (long neck clams), *Ensis americana* (razor clams), *Mactra solidissima* (large sea clams), *Pecten irradians* (scallops), *Mytilus edulis* (mussels), *Modiola modiolus* (mussels), *Fulgur canaliculatus* (winkles, whelks), and *Nassa obsoleta*. The number of specimens of each species examined and the positive findings of *Cristispira* and styles are shown in Table II.

As the tabulation shows, *Cristispira* was found most frequently in the styles of oysters, next in clams, and then in modiolas. No other species examined showed any *Cristispira*. In handling the

styles of these shellfish it was observed that those of oysters, although moderately solid, became liquefied into a viscid fluid upon standing for half an hour at room temperature in a small amount of sea water after removal. The styles of modiolas are much smaller than those of oysters and somewhat firmer; those of clams are much more solid and elastic than either of the others and did not become liquefied on exposure to air and sea water at room temperature. Scallops have large and firm styles which remain little changed after removal. Styles from the other varieties are very small and solid. The physical properties of the styles may be partly or chiefly responsible for the presence or absence of *Cristispiræ* in these shellfish; apparently the consistency of the styles of oysters offers the optimum conditions for the habitation of the organism.

Another striking fact is that, at least in the case of the oysters, the longer they were kept in a tank (eel pond) or the car (sea) after collection from their original beds, the less frequently they contained styles. In such specimens the presence of *Cristispira* is also less frequent, and in many of them only degenerated forms may be found. It is rather easy to determine the freshness of oysters by examining them for styles and the activity of the cristispira contained in them; even freshly collected oysters soon lose the styles if they are not extracted immediately. In one instance 48 oysters which had been kept in the eel pond 24 hours after collection were opened one after another and left at room temperature for about 30 minutes, when they were searched for styles, but unsuccessfully. Six more oysters from the same lot which had not been opened all had perfect styles, and five contained *Cristispira*. These external factors must be taken into consideration in making a survey as to the frequency of *Cristispira* infection of oysters. This rule does not apply to those shellfish whose styles are firm and resistant to manipulation.

The main object of the present work was to study the conditions under which these coarse spiral organisms can be kept alive or cultivated on artificial media, in order that their biological and morphological characteristics might be better studied. Experiments were also made in which the organisms were subjected to the action of various salts, acids, and alkalies in order to obtain an insight into the finer

structure of the organism. Vital stainings with neutral red, crystal violet, brilliant cresyl blue, methyl green, methyl orange, Bismarck brown, and Janus green were also applied.

Dark-Field Examination of Styles.

Oysters.—The style from the oyster is a glassy, somewhat elastic, cylindrical body of about 2 to 3 cm. long and about 3 mm. in diameter at the thickest portion, tapering to a point at the end. When placed in a Petri dish it becomes a sticky viscid fluid; it dissolves in sea water. When a drop of the liquefied style is examined under the dark-field microscope there are observed in many instances violently motile organisms of large size moving through the field with great velocity. They pass out of the field so rapidly that their exact form is not at once recognizable; they suggest butterflies constantly flapping luminous wings; their movements are backward as well as forward. They become sluggish within a short time and come to rest either in a tortuous form or stretched out. The body is a long cylinder with blunt ends. The end portion is slightly thinner than the rest, but not sharply drawn. There is a thick refractive margin all along the body, indicating the presence of definite peripheral concentration of the protoplasma (perhaps a cell membrane). The inner part of the body seems to present no distinct transverse partitions (so called chambered structure), but there are rows of refractive projections from both sides of the wall, point to point, as if they were imperfect cross-bars; the intervals between the bars are about 3 μ. Along the entire body runs a peculiar undulatory narrow membranous structure coiling obliquely and spirally, showing a free edge here and there (known as the crista, or ridge, of the cristispira). It follows a wavy course, and at each wave summit the light is highly refracted. From that point innumerable fibrillar, fan-like radiations run into the body of the organism upon which the other edge of the membranous structure rests or is in connection (Figs. 1 to 3). The fibrils along the free edge of the crista seem to be so arranged as to form a more compact structure than the portion which stretches between the edge and body of the organism. Locomotion appears to be accomplished by alternate bending and relaxation of the undulatory, fibrillar, highly elastic membrane, as is easily demonstrated in a specimen whose

motility is slackening because of exhaustion. In a later period of exhaustion the organism becomes immotile, and the elastic wavy frame of the membrane assumes its full length and shape, causing the body to conform to its spirals (Fig. 8). Only active specimens take the shape of a snake (Figs. 1 to 3). In a freshly extracted style which is still unsoftened by exposure to the air we often find a nest of organisms in one field (Figs. 4 and 5), some assuming positions like coiled snakes, and some rather regularly waved and stretched out. In a few moments the former move rapidly from their resting places with a violent locomotion and swim away; the latter, which represent skeletons or elastic frames (Fig. 8), are degenerated and remain immotile. When a liquefied style containing active specimens of the organism is left for several hours under unfavorable conditions (room) most of the organisms undergo degeneration and are found in various stages from merely stretched out, regularly wavy organisms to skeletons without the body (Figs. 6, 7, and 9). The disintegration of the body is shown by an irregular contour and various degrees of thickness of the body substance. The skeleton often shows clearly its component elastic fibrils, some of which may take the shape of a bushy horse tail (Fig. 6). The gradual overpowering of the body by the elastic membrane in a dying specimen is shown in Figs. 7 and 9.

Clams.—The styles of clams are thicker, firmer, and somewhat longer than those of oysters, faintly yellowish, shiny, somewhat opaque, and do not undergo liquefaction in sea water when removed to a Petri dish. For dark-field examination it is necessary to macerate them in a mortar in order to obtain an emulsion. Five only, of 110 styles examined, showed the presence of active *Cristispiræ*; 3 others contained degenerated specimens. The dark-field appearance of the cristispiras found in clams was identical with that of the cristispiras in oysters (Fig. 10).

Modiolas.—The modiolas are small mussels, with proportionately small vitreous styles, not longer than 1 cm.; the consistency of the styles is softer than that of clams but firmer than that of oysters. For dark-field examination they can be easily macerated on the slide by pressing under a cover-glass. *Cristispiræ* were present in 4 of 97 styles studied. The organism showed microscopically no distinctive features except that of exceptional length (Fig. 11).

Examination by Means of Vital Staining.

The cristispiras from oysters were used for this purpose.

Neutral Red.—Within a few minutes the body showed a general brownish yellow coloration, with numerous paired yellowish granules scattered with fair regularity along the entire wall. The membrane, or crista, appeared as bushy brownish fibrils projecting in all directions. The color of the body disappeared in 20 minutes, that of the fibrils in 6 hours. Coiled specimens did not take the stain.

Crystal Violet.—The body took a rather deep purple stain, the crista remaining uncolored. Fine protoplasmic reticula with intersecting granules were seen. In some specimens vacuolation of the cell contents occurred, a large mass of substance occasionally protruding into the side (plasmolysis). The deep purple color was still present at the end of 6 hours. There was no chambered appearance of the body.

Brilliant Cresyl Blue.—The body took up in mottled fashion a light bluish lavender color. The cross-bars (bluish) of the body were distinctly brought out in a few minutes; the crista remained practically unstained. Complete plasmolysis and decoloration occurred within 6 hours.

Methyl Green.—The body stained faint pink without showing any structure; the crista was not stained. Decoloration occurred in 6 hours.

Methyl Orange.—There was no coloration. The organisms remained active.

Bismarck Brown.—The body became distinctly brownish, with more deeply stained granules throughout the entire length. These granules sometimes occurred in a line along one or both sides of the wall. Degenerated specimens were not well stained. The specimens were practically decolorized in 6 hours.

Janus Green.—The body took a deep, dark bluish color, with reticular mottles, the crista a dark blue on its free margin but only a faint blue elsewhere. In some specimens comparatively large round granules of varying size, stained dark bluish, were seen scattered about in several parts of the body. Degenerated organisms did not take the stain. Within 6 hours the color gradually faded.

In general, then, the body of *Cristispira balbianii* took the vital staining, while the crista stained only slightly with some of the dyes and not at all with others. The phenomenon common to staining with neutral red, brilliant cresyl blue, Bismarck brown, and Janus green was the fact that within the first 5 minutes the body of the organism took the stains in such a way as to show the presence of chromophil granules more or less regularly and diametrically paired on both sides of the wall. After a longer period the number of chromophil granules became more numerous, and a sort of reticular distribution of the same substance was brought out, giving the entire body a mottled appearance. In the case of brilliant cresyl blue the cross-bar arrangement of the chromophil substance was distinct but this was followed by complete plasmolysis of the organism, indicating that the chambers were the result of an unfavorable or toxic effect of the dye. Coiled specimens usually showed no deeply staining granules with Bismarck brown or neutral red.

Stained Preparations.

Several methods were used for the study of the morphological characteristics of the cristispiras, but the best differentiation of the various constituents of the organism was obtained by application of Giemsa's stain and Heidenhain's iron-hematoxylin.

With Giemsa's stain the organisms were variously fixed. In some instances the liquefied styles containing the cristispiras were thinly spread over a clean slide and the moist film surface was immediately exposed to osmic acid vapor (1 per cent) for 1 minute. 30 minutes fixation in absolute alcohol followed. In other instances the moist films were at once immersed in a jar containing Schaudinn's sublimate alcohol, which was maintained at a temperature of 75°C. for 5 minutes and then allowed to cool. Before applying Giemsa's solution the films were rinsed in water, treated first with Lugol's solution, then with 0.5 per cent sodium thiosulfate solution, then thoroughly washed in water. In this procedure the films were never allowed to dry until after being stained with Giemsa's solution. In still other instances the films were first dried in the air, then fixed in methyl alcohol for 30 minutes, then stained with Giemsa's solution.

The object of these various procedures was to study the influence of fixation on the morphology of the organism.

The morphological features revealed by Giemsa's stain were very similar, whether the films were fixed by the osmic vapor or by Schaudinn's hot sublimate alcohol method. In both preparations the body showed a cross-bar structure consisting of deeply stained blue bars alternating with light, almost unstained or faintly pinkish areas. The bars, which were correspondingly wider in larger specimens, appeared somewhat narrower toward the extremities. The relative width of the bluish bar and of the juxtaposed light spaces was very variable in different specimens. In some the width was nearly the same (Figs. 29, 30, and 40); in others the light spaces were much wider than the cross-bars, which then appeared like thin cross lines (Figs. 12, 13, and 31); in still others the reverse was true, in which case a deeply stained bluish body seemed to be segmented by narrow light cross spaces (Figs. 14, 15, 17, and 32 to 34). In the specimens fixed in sublimate alcohol there were specimens showing in some portion of the body several consecutive bars, so thinned out near the the middle as to be almost broken (Figs. 13, 17, and 35), the appearance being not unlike that of the organism in the fresh state. The bluish cross-bars were not of uniform width or sharply defined borders, but one end was usually broader than the other, and sometimes a few here and there were placed obliquely to the right angle formed by the wall and the hypothetical axis of the body. In contrast to fresh specimens, the stained organism generally had a tapering form, ending in sharply pointed extremities (Figs. 12 to 14, and 29 to 35). The crista took a brilliant red hue, showing one or more heavily stained marginal fibers, and it ended near the end of the body (Figs. 29 to 35). The connection between the body and the heavy marginal fibers of the crista is so thin that the finer fibrillar structure is seldom brought out. One may encounter in stained preparations specimens caught in full activity (Figs. 12, 14, 15, 17, 19, 29 to 33, and 35) or in the exhausted condition, yielding to the elastic crista, with beginning plasmolysis (Figs. 20 and 21) or with the crista skeleton (Figs. 23 and 36). Sometimes the crista alone may be found (Figs. 37 to 39).

The specimens fixed in methyl alcohol after being air-dried did not show so sharp a structure as those just described. The organism

stained more reddish in general, and the bluish bars were not clearly distinguished (Figs. 16, 18, and 19).

Heidenhain's iron-hematoxylin stain was applied to film preparations of the cristispira derived from clams and Mallory's to sections of oyster styles. In the latter the organisms were present in large numbers, usually lying parallel with the course of protein lamellæ, which run concentrically along the long axis of the style like a scallion. Some were coiled, but most assumed wavy forms (Fig. 22). The cross-bar structure was easily distinguished. The crista is occasionally revealed—it is perhaps held closely to the body. (In fresh specimens the crista can always be seen.) Occasionally there are found specimens cut across so that they are visible as a ring. The details of structure are more clearly brought out in the film preparations by adequate differentiation (Figs. 25, 27, and 44). The body is cross-barred by thin, dark grayish lines, with a more definite contour of the cell wall. The crista is fibrillar and retains a light bluish gray color which is deeper along the marginal fibers. The extremities of the body are not sharply drawn out but are blunt points.

In one of the clams collected in New York Bay in September, 1916, I encountered a rather long variety of *Cristispira*. It was similar in all other respects to the variety met with in the clams studied at Woods Hole. Whether this specimen of *Cristispira* is a new species or merely a variety due to temporary factors has not been determined (Figs. 24, 26, 28, 45, and 46).

Effects of Chemicals.

The microchemical reactions of various components of *Cristispira balbianii* were studied with a view to gaining an insight into the structure of the organism. Previous investigators have obtained varying results in a similar study of the European specimens. The writer's studies with the American variety were made with the following substances: sodium taurocholate, sodium glycocholate, sodium oleate, saponin, cobra lecithid, acetic acid, hydrochloric acid, sulfuric acid, potassium hydroxide, and ammonia.

Method of Study.—One part of the style emulsion containing numerous active specimens of *Cristispira balbianii* was mixed on a coverglass with one part of the substance, dissolved in sterile sea water;

TABLE III.

Effect of Chemicals on Cristispira.

Date.	Chemical.	Concentration.	Result.
1916		*per cent*	
Aug. 23	Sodium taurocholate.	10	Plasmolysis complete in 5 to 10 min. Skeleton, or crista, resistant. *Spirillum ostreæ* dissolved; bacilli still motile. After 20 hrs. at room temperature skeletons (cristas) showing their fine fibrillar composition are more completely denuded of the cell plasma.
		1	Practically the same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis; otherwise the same as preceding experiment.
	Sodium glycocholate.	10 1 0.1	Similar in effect to the taurocholate solution.
	Sodium oleate.	10 1 0.1	This salt produced such a viscid emulsion that no observation was possible.
	Cobra lecithid.		Almost insoluble in sea water. Observations indecisive.
	Saponin.	10	Complete plasmolysis in 5 to 10 min. Crista not affected but assumes more regularly waved spiral bundles of fibrils (Fig. 9), to which irregularly protruding masses of protoplasm are seen to be attached at some points. *Spirillum ostreæ* and bacilli still motile. After 20 hrs. at room temperature the cristas are still intact. They show their finer structure and their relation to the bodies, which are now mere masses of broken up protoplasm scattered along the regularly undulated spiral cristas.
		1	Same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis.
	Controls in sea water.		Actively motile for 2 hrs. after being sealed on slides; thereafter gradually became immotile; structure unchanged.
			After 20 hrs. no degeneration but all in "relaxed" state.

TABLE III—*Concluded.*

Date.	Chemical.	Concentration.	Result.
1916		*per cent*	
Aug. 24	Acetic acid.	50	After 2 hrs. body smooth, showing cross-bar structure and heavy cell wall; crista shrivelled and indistinct; *Spirillum ostreæ* swollen and indistinct.
		10	Same as with 50 per cent.
		1	" save for the presence of many round bodies suggesting extruded protoplasm of the body of *Cristispira balbianii*.
	Hydrochloric acid.	50	After 2 hrs. body shows more densely set cross-bar appearance than in acetic acid, but the crista, though perhaps thickened and less distinct than normally, is still attached to the body.
		10	Same as with 50 per cent.
		1	" except for some nodular swelling of the body in parts.
	Sulfuric acid.	50	Similar to results with hydrochloric acid except for more granular appearance of the body substance and less distinctness of the crista.
	Potassium hydroxide.	15	Within 2 hrs. the organisms have practically disappeared, except for a few fragments of the bodies in which no structure save the cell wall can be recognized; no crista found.
		3	Within 2 hrs. the body shows irregularly contoured thick wall with vestiges of thinned out, broken cross-bars; crista seems to be closely attached to the body.
		0.3	Within 2 hrs. there are apparently no changes; body shows distinct cross-bars and heavy cell wall; crista seems to be intact.
	Liquor ammoniæ fortis.	50	Within 2 hrs. no changes; immobilized.
		10	" 2 " " " "
		1	" 2 " " " "
	Controls in sea water.		After 2 hrs. almost all are still active.

the cover-glass was then sealed with vaseline on a slide and examination made under the dark-field microscope. The concentration of each substance and the effects of each upon the organism are briefly summarized in Table III. The observations were made at room temperature (23°C.).

Survival of Cristispira balbianii under Different Conditions.

An oyster emulsion containing actively motile cristispiras was used to study the effects of tonicity. The observations were made in hanging drop preparations after 2 hours at room temperature (22°C.). When mixed with distilled water the organisms became immotile, then the cell body assumed a regularly spiral course in conformity with the skeletal crista, presenting at the same time accumulations of a highly refractive substance in round or oval masses along the body at each turn of the spiral skeleton. The phenomenon may be interpreted as due to plasmolysis through hypotonicity. On the other hand, in 10 per cent sodium chloride solution the organisms remained intact, but many coiled forms (Fig. 40) were seen. The body appeared less refractive, but there was no plasmoptysis. In the control preparations made with sea water, most of the organisms became immotile after 2 hours at room temperature; in those kept at 6–8°C. all the cristispiras were still actively motile at the end of 2 hours, and one-fifth to one-tenth after 24 hours; after 40 hours only a few were found to be active in one of the three slides. In dark-field preparations (not hanging drop method), sealed with paraffin and kept at 6–8°C., all cristispiras were disintegrated at the end of 24 hours.

Attempts at Cultivation.

Several unsuccessful attempts were made to obtain a culture of *Cristispira balbianii*. The chief obstacles in the work were (1) the lack of bacteriological facilities, and (2) the impossibility of obtaining a bacteria-free suspension of *Cristispira balbianii*.

The culture media employed in the first attempts were made with a filtrate of various styles (oysters and scallops), obtained by passing the sea water solution (or emulsion) of styles (50 styles in 100 cc. of sea water) through a Berkefeld filter V. To 3 cc. of the filtrate were

added, in one set, 1 cc. of 2 per cent glucose agar; in another set 1 cc. of 2 per cent plain agar; in a third set 1 cc. of ascitic fluid; in the fourth 1 cc. of glucose broth; in the fifth 1 cc. of sterile sea water; and in the sixth (controls) 4 cc. of sterile sea water without the style filtrate. All tubes were covered with a thin layer of sterile paraffin oil to prevent evaporation of the culture media. The cultures were set up as follows: All tubes (1 cm. in diameter and 22 cm. high) first received 0.2 cc. of a rich cristispira emulsion from oysters. 3 cc. of the style filtrate were then added to all except the control (sixth) set. The first and second sets were next mixed with 1 cc. of 2 per cent melted glucose agar and plain agar, respectively, the temperature of the agar being about 42°C. at the time of mixing. The tubes were cooled quickly by immersion in the aquarium. The glucose broth and ascitic fluid were added to the other sets and the paraffin oil was finally added to all. Each set was made up in duplicate, one being kept at room temperature and the other at aquarium temperature (about 7°C.).

Examination of the culture tubes was made daily during 8 days, but no growth of cristispira was ascertained. They were brought to The Rockefeller Institute in an ice-packed container and followed for 3 weeks longer, but except in one instance, in which a peculiar large non-motile organism was seen to grow, the results were negative. This organism, which grew in the first set of media (semisolid), bore a close resemblance to *Cristispira*, except that there was no crista. It suggested Gross' *Saprospira*. Whether it was a cristispira modified through artificial cultivation (loss of crista) or an altogether different organism is still a question. Most of the tubes were more or less contaminated with members of the *mesentericus* group.

A spiral organism of oysters, *Spirillum ostreæ*, *n. sp.* (Fig. 43), likewise failed to grow. This spiral organism measures about 8 to 16 μ in length and 0.5 μ in the widest portion of the body. Both ends gradually taper off to fine filaments. The number of spirals varies from 4 to 8 and the curves are rather shallow but very regular. The body is elastic but not perceptibly flexible. They rotate rapidly but seldom proceed one way or the other.

In another experiment styles were ground in a mortar with Ringer's or Locke's solutions as well as with sea water and the whole was

sterilized in an autoclave. The opalescent fluids thus obtained were used in experiments similar to those preceding with unsatisfactory results.

The tissue culture technique was employed in order to determine the viability of cristispiras in the presence of various extraneous substances. By careful examination it was possible to collect a dozen styles rich in cristispiras and apparently free from ordinary bacteria. *Spirillum ostreæ* was the only variety present and occurred in rather large numbers in some styles. The styles were dissolved in 3 cc. of sterile Ringer's solution; the work was carried out as aseptically as circumstances permitted.

To 1 drop of the style emulsion was added 1 drop of the following liquids respectively: (1) mixture of oyster style-Locke's solution infusion filtrate; (2) oyster broth-Locke's solution-infusion-style filtrate; (3) oyster juice filtrate; (4) oyster broth; (5) Locke's solution; (6) sea water; (7) distilled water; (8) ascitic fluid; and (9) bouillon. Eight Ringer's solution preparations were made as controls.

At the end of 24 hours at 6–8°C. many *balbianii* remained active in three of the eight control (Ringer's solution) hanging drop slides, and in the slide containing ascitic fluid. In all other instances the organisms were inactive and diminished in numbers; there was an increasing mass of bacteria more marked in the slides containing albuminous solutions like oyster-filtrate, Locke's solution-style infusion, and bouillon. At the end of 48 hours only a few cristispiras were still sluggishly motile in the slides containing ascitic fluid and in one of the Ringer's solution controls.

This observation serves to emphasize the difficulty of obtaining a culture of this organism under the conditions of the experiments just described.

DISCUSSION AND SUMMARY.

Ten varieties of North American shellfish were examined for the occurrence of *Cristispira* in their styles. A cristispira was found in various numbers in *Ostrea virginiana*, *Venus mercenaria*, and *Modiola modiolus*, but none in *Ensis americana*, *Mya arenaria*, *Mactra solidissima*, *Pecten irradians*, *Mytilus edulis*, *Fulgur canaliculatus*, or *Nassa obsoleta*. Of 298 oysters, only 128 showed the crystalline styles, in

which cristispiras were present in 99. Active cristispiras were found in 59 styles only and degenerated forms in the remaining 40. In 110 clams (*Venus mercenaria*) 70 styles were found, and only 8 of these contained cristispiras; 5 yielded active and the other 3 degenerated cristispiras. In 97 modiolas there were 73 styles, only 4 of which contained cristispiras.

The physical properties of the crystalline styles of these shellfish varied considerably. The styles of the oysters were moderately soft, and when exposed to the air or mixed with sea water they underwent liquefaction, forming a clear, viscid material. The styles from clams and modiolas were opaque and were more firm, not easily crushed even in a mortar. The styles of the scallops were the most solid of all the styles examined. It happened that the softer the styles, the more frequent was the occurrence of the cristispira; in fact, no cristispira was detected in styles other than those of oysters, clams, and modiolas, of which oysters had the softest styles and the largest percentage of cristispira invasion.

The following observations were made regarding the structure of the cristispira found in oysters. The body is a long, flexible cylinder, with blunt extremities, towards which the diameter gradually diminishes. In motion the body rapidly stretches and contracts, forming in the contracted state several serpentine undulations. A membranous appendage (Gross' crista) winds about the body throughout its entire length. The inner margin is in connection with the body, the outer margin is free and is distinctly heavier. The latter is undulatory; that is, the width of the membrane, or crista, is narrower at some points than at others. The membrane is composed of numerous fine fibrils running in a roughly parallel or slightly oblique course, showing interwoven narrow meshes; at the outer margin there is a dense smooth ridge.

The contour of the body is highly refractive, as if possessing a cell membrane. The interior structure, as revealed by dark-field illumination, is an almost homogeneous, less refractive substance, but there are present minute highly refractive granules more or less symmetrically arranged. There is no definite cross-bar or chambered structure. On the other hand, when vital staining with brilliant cresyl blue is applied, there appear numerous paired masses of lavender hue at

fairly regular intervals, suggesting the cross-bar aspect of a stained specimen. In a few specimens there was seen a dim outline of cross-bar effect. Neutral red, Bismarck brown, and crystal violet all bring out deeply stained granules and reticular structure but no definite cross-bars.

While it is difficult to recognize any definite chambered structure in the fresh state, it is very easy to bring out the characteristic banded body by staining the organism with Giemsa's solution. The bands, or cross-bars, which take up a deep bluish color, are of variable width, ranging from a thin line to a square or even oblong block. In some specimens the body is almost blue with thin white cross lines, while in others the blank body is banded with thin bluish bands at irregular intervals. The crista, or undulatory membrane, is stained bright red, particularly along the outer free margin. The fibrillar structure of the crista is usually recognizable. In the specimens stained with Heidenhain's iron-hematoxylin the cross bands, which appear bluish gray, are more distinct and delicate. The bands are not, however, always thin and sharp, but in certain specimens are almost square. The irregularity in the width of the cross bands in various parts of one specimen and in different specimens suggests that the peculiar banded appearance may be a result of fixation and not the natural structure of the organism. At least, no such structure was recognized under the dark-field microscope, which would have revealed such definite septa if they existed. I believe that the cross bands are formed from a homogeneous mass through sudden contraction due to dehydration with absolute alcohol during fixation. In the preparations first dried in the air and then fixed with methyl alcohol this banded appearance is absent. That *Cristispira* has a chambered body, each chamber being equivalent to a single cell, is Gross' interpretation, which is not supported by the observations reported here. Moreover, the protoplasm of the organism readily escapes from the body in distilled water, saponin, or other chemical agents, forming many round masses of extruded protoplasm in different parts of the body. No septa are to be seen in these plasmolyzed or degenerated organisms. Acids, hydrochloric and acetic, failed to bring out any banded structure of the body. It may be deduced that the external surface of the body is a highly refractive cell membrane, and that

within the cell body are homogeneous protoplasm of reticular form and numerous highly refractive granules of varying sizes. Some of the contents take the dark bluish color when stained with Giemsa's solution. In a fixed specimen this particular substance becomes contracted into a series of masses of varying dimension, leaving between each mass a blank space of varying width, and giving a characteristic cross-barred or banded (so called chambered) structure. In some specimens the cross-bars are not seen, but instead a series of broken lines running parallel with the longitudinal axis of the body (Figs. 41 and 42).

The crista is a fibrillar structure, connected with the body at its inner edge. The outer margin is a thickened bundle of fibrils running an undulating course along the entire length of the crista. The crista is elastic and when detached from degenerated organisms assumes a rather regularly wound spiral, consisting of longitudinal bundles of fibrils (Figs. 36 to 38). A fragment of two or three waves may be encountered in a preparation containing many degenerated organisms (Fig. 39). The composition of the crista can best be studied in degenerated remains of the organism. During the life of *Cristispira* it is stretched or relaxed according to the contraction or extension of the body. The elasticity of the crista appears to furnish the organism with a propelling and rotating power upon its extension after being drawn tightly to the body by some contractile apparatus (myoneme) present somewhere within the cell body. The crista serves as a rudder and propeller for the swimming organism. It is interesting to compare here the elastic and regularly waved flagella of certain bacteria and spirochetes; it is possible that the crista of *Cristispira* is a highly modified form of flagella.

The nature of the substance which stains dark blue with Giemsa's stain is not known, but it does not give a chromatin reaction. By Heidenhain's iron-hematoxylin method it takes a dark grayish tint, similar to the cell wall or crista, which are also dark gray. This substance was regarded by Gross and Zuelzer as volutin, which is of nutritive origin. It is probable that there are also embedded within it minute chromidial elements. Multiplication is by transverse fission.

Cristispira balbianii is parasitic and does not survive more than a few days in ordinary sea water emulsion, even at its optimum

temperature. In its natural habitat, or the crystalline style, it is usually pure, but is sometimes found in association with a tiny spiral organism (*Spirillum ostreæ*). The cristispiras in the styles seem to diminish rapidly when oysters are collected from their beds and transferred elsewhere; oysters kept in tanks or cars for several days do not contain the cristispiras, and in opened oysters the styles disappear promptly at room temperature.

All efforts to cultivate this organism have failed.

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EXPLANATION OF PLATES.

PLATE 21.

Magnification uniformly × 1,000.

FIGS. 1 to 3. Dark-field view of living specimens of *Cristispira balbianii* from an oyster. Photographed while they were temporarily at rest. The smooth wavy body with sharp contour along the wall, and a somewhat hazy alveolar content are well shown. The undulating crista loosely attached to the body of the organism can be seen. What appear like pointed sticks attached to the organism in Figs. 1 and 3 are the spirillar organisms found in fresh oysters which were merely entangled in the crista.

FIGS. 4 and 5. Dark-field view of *Cristispira balbianii* in the styles of oysters. In the styles there are different forms undoubtedly representing various stages of the life of the organism. In Fig. 4, there are one degenerated and two well preserved specimens, while in Fig. 5 there are also two intact and one long skeleton of a degenerated organism.

FIGS. 6 to 9. Different phases of degeneration of *Cristispira balbianii* due to unfavorable conditions or to age. Fig. 9 shows the protoplasm bulb accumulated near the middle of the body under the influence of a concentrated solution of saponin. Figs. 6 and 8 show the elementary fibrils which constitute the crista.

FIG. 10. Dark-field view of a cristispira from the style of a clam. In every respect this organism is comparable to the *balbianii* found in oysters. It may be the same as that described by Dobell as *Cristispira veneris*.

FIG. 11. Dark-field view of a cristispira from the style of a modiola. Generally somewhat longer than the *balbianii;* otherwise appears identical.

PLATE 22.

Magnification uniformly × 1,000.

FIGS. 12 to 15 and 17. *Cristispira balbianii* from oysters, stained with Giemsa's solution after osmic acid or sublimate alcohol fixation. They are all well preserved.

FIGS. 16, 18, and 19. *Cristispira balbianii* stained with Giemsa's solution after methyl alcohol fixation. All except Fig. 16 appear in good condition, although no definite cross-bar structure can be distinguished.

FIGS. 20, 21, and 23. *Cristispira balbianii* in process of degeneration. Fig. 20 shows the body still solid, almost intact, with a regularly wavy crista. In Fig. 21 the body is still fairly well preserved, but it follows the regular waves of the crista, indicating the absence of any resistance to the form of the crista. Note also the protruding mass of protoplasm near the middle of the body. In Fig. 23 there is only a shadowy trace of the body and the spiral elastic skeleton, or crista, is distinct.

FIG. 22. A section of the oyster style fixed in sublimate alcohol and stained with Mallory's iron-hematoxylin. The organisms show the chambered structure, but no crista can be distinguished.

FIGS. 25 and 27. *Cristispira balbianii* in films from fixation sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

FIGS. 24, 26, and 28. Cristispira from a clam caught in New York Bay. The film was fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

PLATE 23.

Magnification $\times 1{,}250$.

Drawings from film preparations fixed in osmic acid vapor and absolute alcohol and stained with Giemsa's solution.

FIGS. 29 to 42. Different specimens of *Cristispira balbianii* showing the general chambered or cross-barred appearance of the body and undulatory membranous appendage, called the crista by Gross. The blue-stained substance which constitutes the cross-bars, or partitions, is seen to be regularly or sometimes irregularly distributed in amount or distance. In some, the substance may not be attached to the wall at all, but forms longitudinal rods of varying lengths as seen in Figs. 41 and 42. In such instances there is no suggestion of a chambered structure.

FIGS. 36 to 39. The final steps of degeneration of *Cristispira balbianii*. Fig. 36 alone still shows the degenerated body while the others show nothing but the fibrous skeletons, or cristas, of the organism. Fig. 39 is a fragment of the crista.

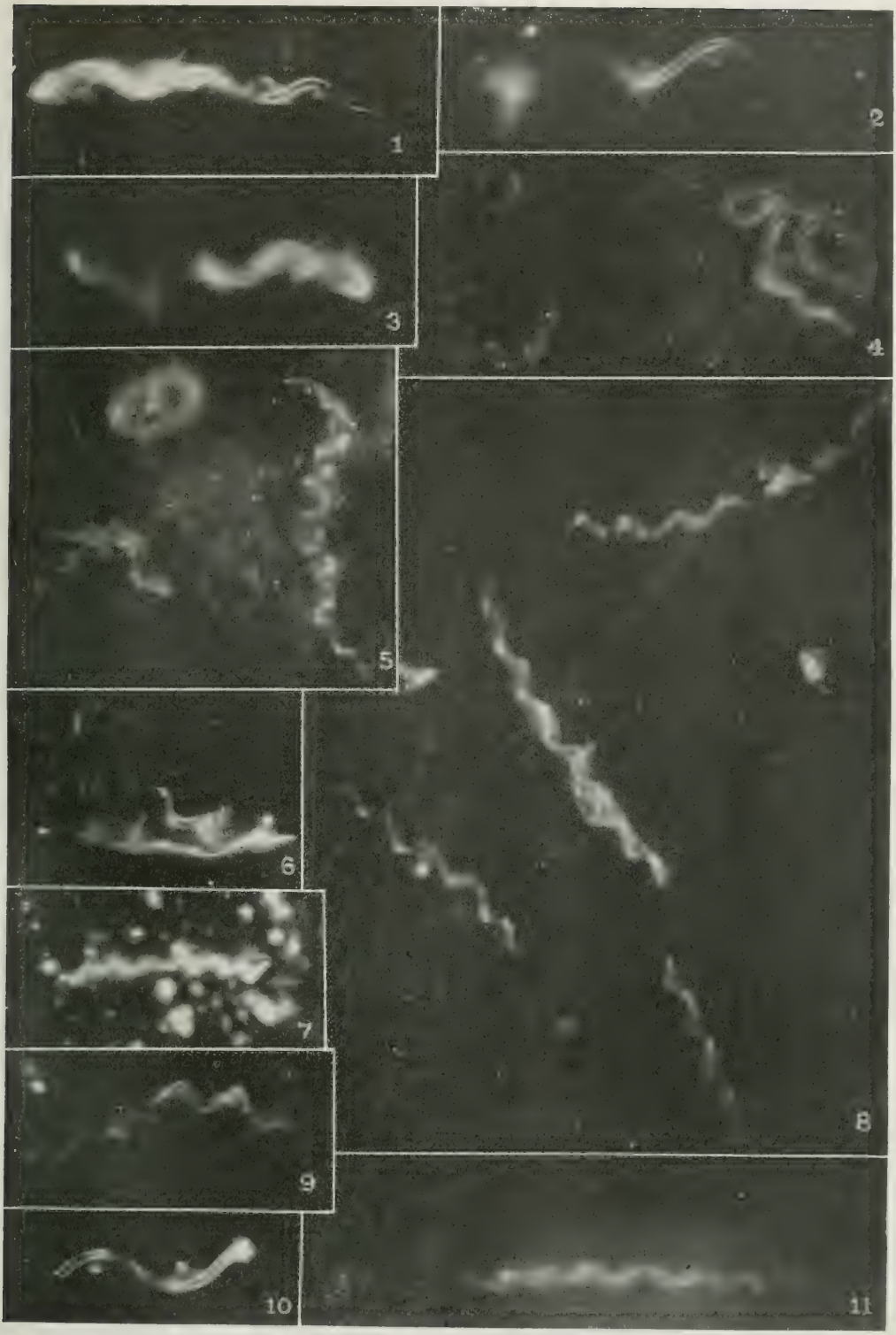
FIG. 43. The spiral organism found in the crystalline styles of fresh oysters caught in Woods Hole Bay.

PLATE 24.

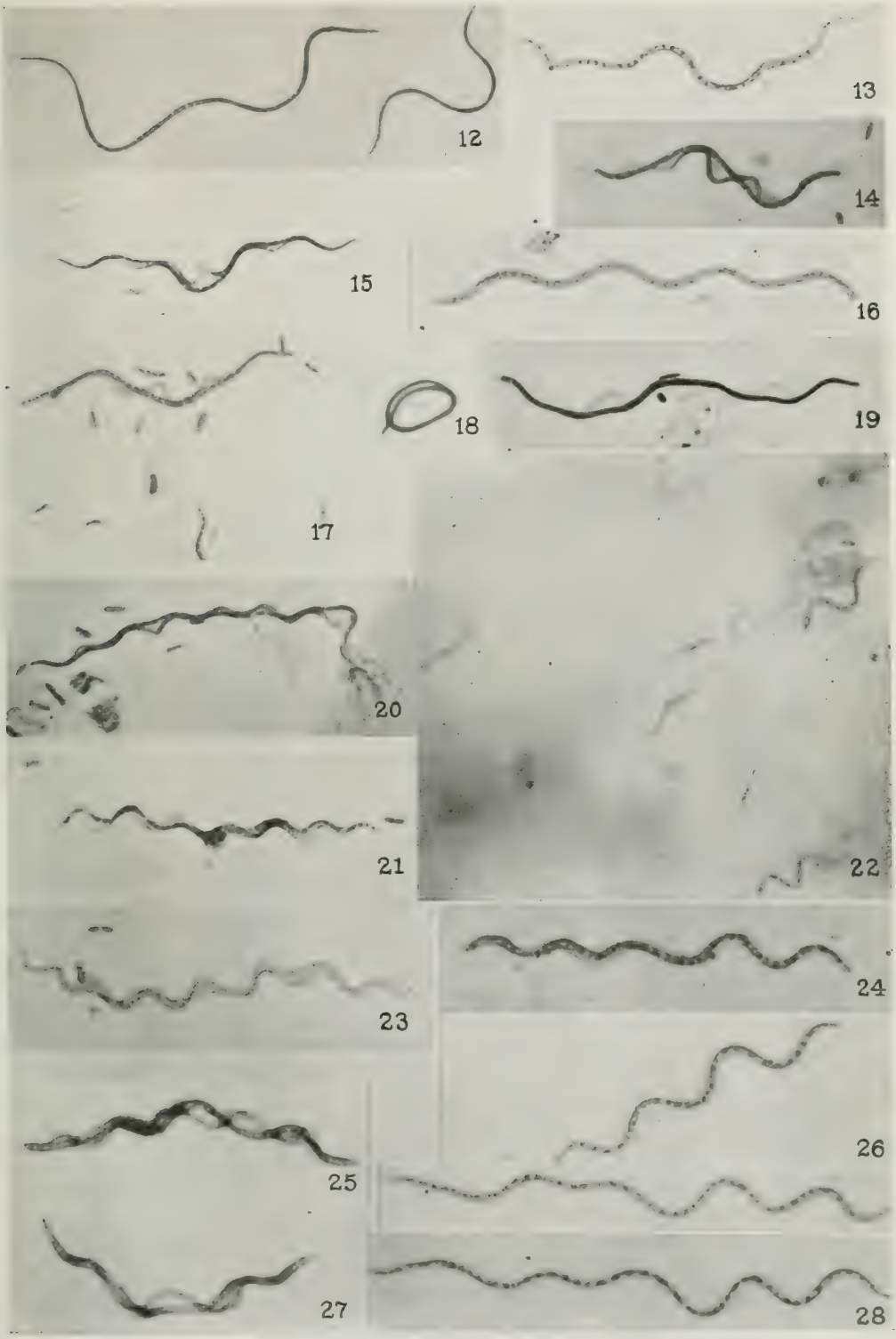
Magnification $\times 1{,}250$.

FIG. 44. *Cristispira balbianii* from an oyster. Film preparation, fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

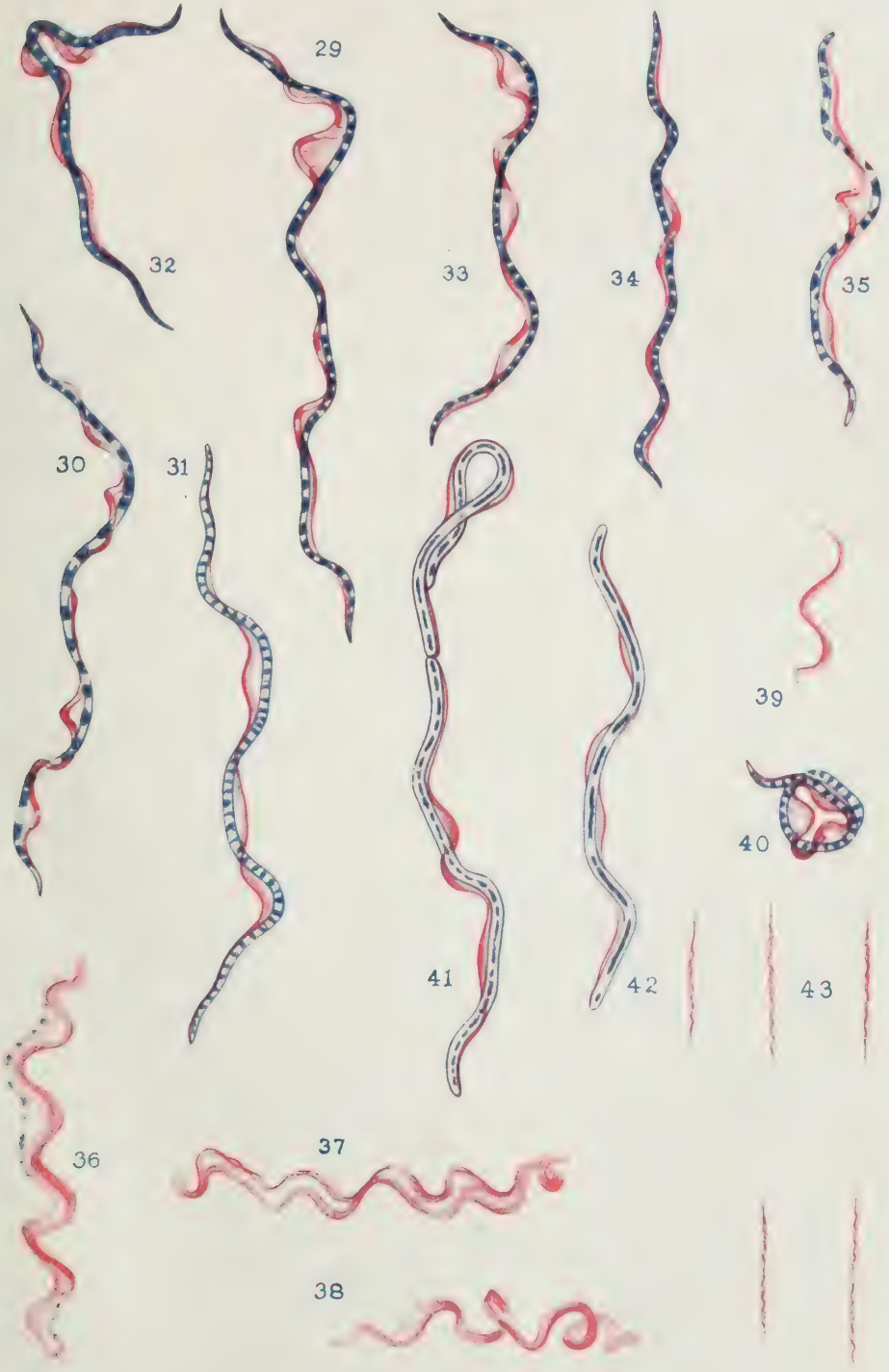
FIGS. 45 and 46. A cristispira (*Cristispira veneris?*) from a clam collected near New York Bay. Sublimate alcohol fixation and Heidenhain's iron-hematoxylin.



(Noguchi: *Cristispira* in North American shellfish.)



(Noguchi: *Cristispira* in North American shellfish.)



(Noguchi: *Cristispira* in North American shellfish.)

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(Noguchi: *Cristispira* in North American shellfish.)

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TYPHUS FEVER AMONG RECENT IMMIGRANTS.

Experimental Demonstration of the Identity of This Disease with European Epidemic Typhus Fever.

By PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plate 25.

(Received for publication, May 20, 1921.)

In January, 1921, through the kindness of Dr. S. B. Wolbach of Harvard Medical School, we received guinea pigs in which two strains of typhus virus were being propagated. One of these strains was originally derived from the blood taken from a patient during the height of typhus fever; the other, from the saline solution washings of a cage harboring typhus-infected lice. In July, 1920, with these materials, Dr. Wolbach, working in Warsaw, Poland, induced experimental typhus fever in guinea pigs; in the next half year he transmitted it through about fifteen successive animals and determined the existence of a cross-immunity between the human and louse strains.

In February, 1921, when we were engaged in an experimental study of the virus of typhus fever, employing for this purpose the guinea pigs given to us by Dr. Wolbach, a number of immigrants arrived at the Port of New York from different parts of Europe and showed a syndrome of headache, continued fever, petechial rash, positive Weil-Felix reaction[1,2] and infestation with body lice, which was suggestive of typhus fever. Through the kindness of Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, we were enabled

[1] Weil, E., and Felix, A., *Wein. klin. Woch.*, 1916, xxix, 974; 1917, xxx, 1511. This test is based on the observation of Wilson (Wilson, W. J., *J. Hyg.*, 1909, ix, 332; 1910, x, 155) that agglutinins for intestinal, colon-like bacilli are present in the serum of typhus fever patients.

[2] Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, applied the Weil-Felix test to these patients and found definite agglutination reactions in all—indeed, in some instances in dilutions of 1: 1,280.

to obtain blood from an immigrant suspected of having typhus fever. We inoculated the blood into guinea pigs in which was set up an experimental disease which we were thus able to compare with the experimental disease being propagated in these animals with the Polish virus.

Herewith is presented a detailed study of the experimental disease induced in the guinea pig by injecting the blood from this immigrant and the results of a comparison of this experimental disease with that set up in the same animal with the human and louse strains of the Polish virus.

Source of Material.

The patient from whom material was collected for the following experiments was a young, adult female, a native of Czecho-Slovakia, who had embarked for New York at Trieste, Italy. At the time of examination she had been ill for 8 days with headache and continued fever. Her face showed the suffusion and her conjunctivæ the injection characteristic of typhus fever, and the anterior surfaces of her forearms and arms were covered with a fading petechial rash. The Weil-Felix reaction, performed by Dr. Holt-Harris, was positive in a serum dilution of 1:320. Body lice infested the patient before admission to the hospital.

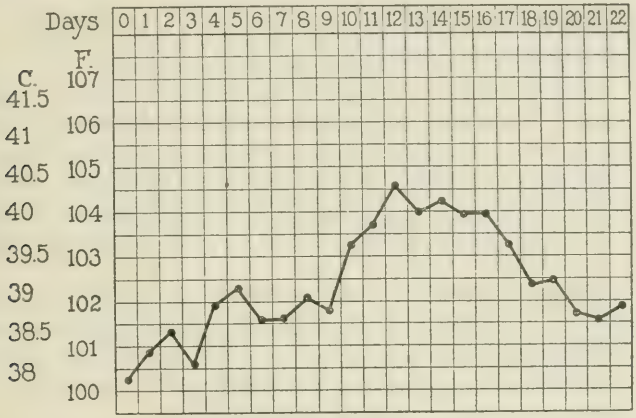
Transmission to Guinea Pigs.

Blood was obtained on the 8th day of illness, by venous puncture with a sterile syringe previously washed in 50 per cent sodium citrate solution, and 3 cc. of the blood, which proved sterile by aerobic cultivation tests, were immediately injected intraperitoneally into each of two guinea pigs, A and B.

Protocol 1.—Guinea Pig A, after 19 days during which the average temperature was 38.5°C., showed on the 20th day a rise of temperature to 40.4°C. It was then bled by heart puncture with a needle and syringe prepared in the usual manner and 3 cc. of the blood, sterile by aerobic tests, were injected into each of three normal guinea pigs. The rise of temperature in the second series occurred, on an average, 11 days after injection, when the blood, in the same amount, was transferred to a third group. In this way we have succeeded in transmitting the fever through at least nine successive animals, with an average incubation period of 9 days.

Guinea Pig B showed a rise in temperature to 40.4°C. on the 7th day after injection, when it was bled and the blood transferred to three normal animals. The guinea pigs of this and subsequent passages (second to fourth) yielded profuse growths of *B. proteus* from the heart's blood and this series was, therefore, discontinued and the results were discarded.

The febrile reaction in the guinea pigs which were allowed to recover lasted from 4 to 8 days. Thereafter the temperature promptly fell to normal and the animals remained well (Text-fig. 1).



TEXT-FIG. 1. Fifth guinea pig passage of the disease induced by the blood of the immigrant. Incubation period 9 days; fever 8 days.

The guinea pigs killed during the febrile reaction showed the following pathological picture.

Macroscopic Examination.—All the organs were normal in appearance with the exception of the spleen which was slightly enlarged, darker than normally, and of which the lymphoid follicles were prominent and raised above the surface. Occasionally the spleen was not enlarged. The skin, because of its hairiness, showed no definite lesions on its outer surface. The inner surface, however, especially after the corium had been stripped off, revealed occasional petechial spots.

Microscopic Examination.—The central nervous system showed characteristic lesions. There was no meningeal involvement except in the case of its blood vessels, which showed the changes described below. The gray matter of the brain, especially that of the midbrain, contained many small, localized hemorrhages. The striking changes were microscopic and consisted of focal accumulations of cells about the capillaries and arterioles. These lesions have been described in

detail by Ceelen,[3] Nicol,[4] and others in European typhus fever, by Doerr[5] and others in the experimental disease in guinea pigs, and recently by Wolbach and Todd[6] in Mexican typhus fever. The nodules consisted mainly of macrophages, or endothelial leucocytes of Mallory,[7] and a few leucocytes, mononuclears, and polymorphonuclears, and were always to be found in close proximity to the vessels. The vessels were surrounded by a more or less dense collar of the macrophages or endothelial leucocytes and leucocytes of the mononuclear and polynuclear type, which at some points was sufficient to form a distinct nodular mass giving rise to the *periarteriolitis nodosa* described by Nicol[4] (Fig. 1). The endothelium of the vessels, often markedly swollen, was necrotic in some portions and proliferated in others; at times the endothelial cells filled the lumen. In other arterioles and capillaries occluding thrombi were sometimes demonstrable.

The heart tissue showed the vascular lesions described by Nicol:[4] localized necrosis of the intima, swelling and proliferation of endothelial cells, thrombus formation, perivascular accumulations of endothelial leucocytes, polymorphonuclears, and mononuclears to form, in some areas, a *periarteriolitis nodosa*, focal nodules of these cells appearing in the interfibrillary spaces usually in proximity to the vessels. Besides, there were a number of small localized hemorrhages (Fig. 2).

The structure of the spleen obscured the vascular picture, but the organ showed, as a rule, congestion and leucocytic infiltration of the splenic pulp, as noted by Nicol.[4] The lymphoid follicles were enlarged and at the center a number of endothelial leucocytes were noted in active phagocytosis, a picture not unlike that seen by Mallory[8] in Peyer's patches in typhoid fever.

The skin showed in the corium nodular areas similar to those found in the brain and heart. These were near the capillaries and arterioles which showed the changes in the endothelium and the thrombus formation described above. In addition there were small, localized hemorrhages (Fig. 3). Similar vascular lesions in the skin of man have been described by Fraenkel,[9] Aschoff,[10] von Chiari,[11] and others in cases of typhus fever in Europe, by Wolbach and Todd[6] in those in Mexico, and by Löwy[12] in experimental typhus fever of guinea pigs.

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[11] von Chiari, R. F., *Wien. klin. Woch.*, 1917, xxx, 1479.

[12] Löwy, O., *Wien. klin. Woch.*, 1916, xxix, 547.

The kidney, adrenals, liver, and testicles revealed no remarkable changes other than the characteristic microscopic nodular formation and the vascular changes. The lesions in these organs correspond to those found in man, in the kidney by Aschoff,[10] in the adrenals by Nicol,[4] in the liver by Ceelen,[3, 13] and in the testicles by Schmorl.[14]

To summarize the results of the experiments described above, it is shown that the blood of the patient at the Quarantine Station induced in guinea pigs a characteristic febrile reaction of from 4 to 8 days duration, preceded by an average incubation period of 9 days. The disease of which this is the expression could be transmitted through at least nine successive animals. Furthermore, the guinea pigs killed during the height of the reaction revealed on microscopic study of the organs constant pathological changes similar to those found by several investigators of typhus fever of man in Europe and Mexico, and of the experimental disease of the same animal.

In these respects, the experimental disease which we have induced in guinea pigs corresponds to that set up in the same species with the human and louse strains of the Polish virus.

Immunity Experiments.

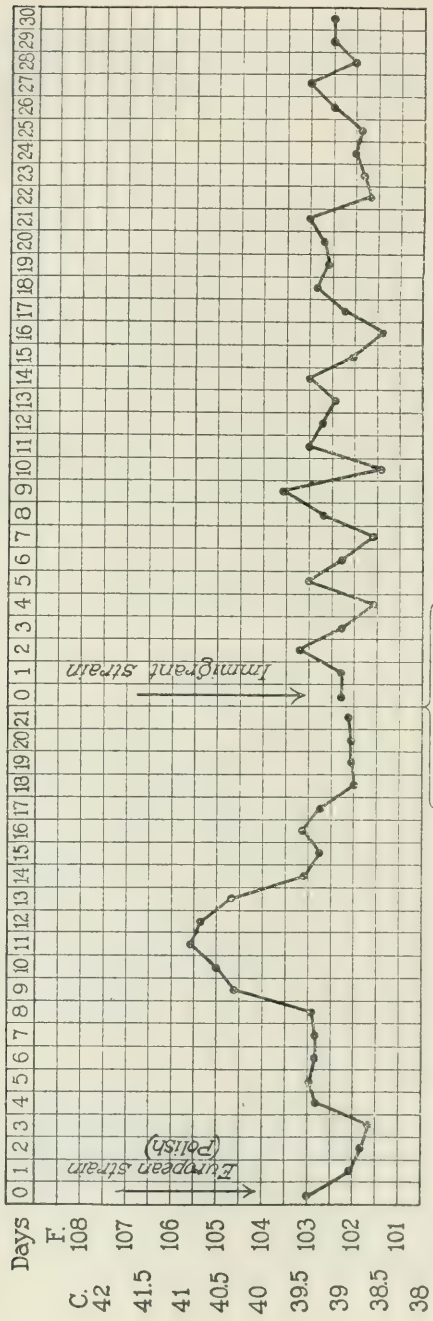
Additional evidence in establishing the identity of the experimental disease in guinea pigs induced with the blood of the immigrant with the disease propagated in these animals with the Polish virus, is afforded by means of the immunity reactions. The study on immunity was made from these view-points: (*a*) the specific immunizing effect of the virus derived originally from the blood of the immigrant when followed by subsequent injections of the active agent of the same disease, and (*b*) cross-immunity between this disease and that of guinea pigs originally infected with the Polish typhus fever virus of man and lice.

The following protocols are illustrative.

Protocol 2.—Guinea Pig B was injected intraperitoneally with 3 cc. of blood from Guinea Pig A, the fourth passage of the experimental disease in this species, derived originally from the blood of the immigrant. After an incubation period

[13] Ceelen, W., *Med. Klin.*, 1916, xii, 402.

[14] Schmorl, C. G., quoted by Nicol.[4]



TEXT-FIG. 2. This guinea pig, after reacting typically to an intraperitoneal injection of the Polish typhus fever virus, human strain, remained immune to a second injection of the immigrant disease.

of 9 days, Guinea Pig B showed a febrile reaction lasting for 6 days; thereafter the temperature was normal. A control guinea pig, injected similarly, reacted with a rise of temperature after 9 days incubation and was killed on the 1st day of the fever. It showed the typical pathological picture already described. Guinea Pig B after 6 days of normal temperature was reinjected intraperitoneally with 3 cc. of the blood from an animal in the sixth passage of the experimental disease. There was no reaction during a period of observation lasting 1 month. A control animal was inoculated at the time of the second injection of Guinea Pig B with similar material. This animal reacted characteristically after an incubation period of 10 days.

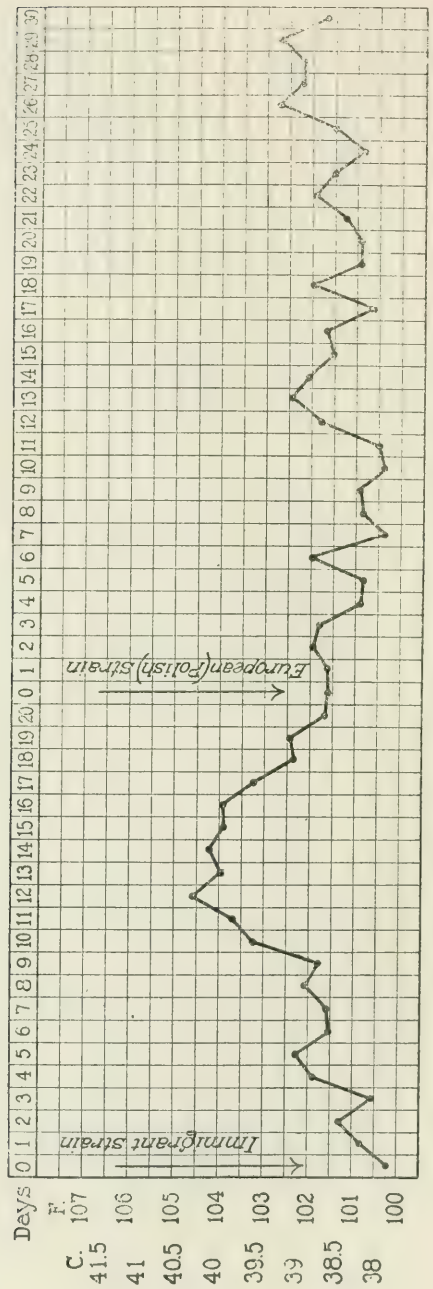
This experiment indicates that after an attack of the disease induced in guinea pigs with the blood of the immigrant, the animal is refractory to subsequent injections of active blood derived through passage from the same patient.

Protocol 3.—The following experiments were controlled in that whenever active blood was employed, it was injected into control animals under the same conditions as the test animal, to determine its clinical and pathological action.

Guinea Pig A (Text-fig. 2) was injected intraperitoneally with 3 cc. of blood from a guinea pig in the nineteenth passage of the experimental disease induced with the Polish strain of typhus virus. After an incubation period of 7 days a rise in temperature occurred, lasting for 5 days. Thereafter the animal showed normal temperature for 19 days, after which it was reinoculated with 3 cc. of blood from a guinea pig in the third passage of the experimental disease induced originally with the blood of the immigrant. There was no effect during a period of observation lasting 1 month.

Guinea Pig B was inoculated similarly, but with the eighteenth passage of the Polish typhus virus, louse strain. A 6 day fever following an 11 day incubation period resulted. 2 days after the fever subsided, the animal was reinjected with 3 cc. of blood from a guinea pig in the second passage of the experimental disease induced with the blood of the immigrant. No reaction occurred although the animal was kept under observation over a month.

Guinea Pigs C (Text-fig. 3) and D had reacted characteristically to injections of blood from guinea pigs in the fourth and fifth passages, respectively, of the experimental disease induced with the blood of the immigrant. Guinea Pig C, 4 days after the fever subsided, was reinjected with the blood from a guinea pig in the twenty-sixth passage of the Polish typhus virus, human strain; Guinea Pig D, 3 days after the fever subsided, with the blood of the twenty-fifth passage of the Polish typhus virus, louse strain. Neither animal was affected during a period of observation of over 1 month.



TEXT-FIG. 3. This guinea pig, after reacting typically to an intraperitoneal injection of the blood from a guinea pig in the fourth passage of the disease originally derived from the immigrant's blood, remained immune to a second injection of Polish typhus fever virus, human strain.

Protocol 3 shows that guinea pigs reacting characteristically to the known strains of typhus virus originally derived from a typhus fever patient in Poland, and from typhus-infected lice collected in that country, fail to respond to a subsequent injection of active blood from guinea pigs with the experimental disease set up with the blood from the Czecho-Slovakian immigrant at the New York Quarantine Station. Conversely, guinea pigs reacting typically to the injections of blood from guinea pigs in the line of transmission of the experimental disease induced with the immigrant's blood, are refractory to later injections of blood containing either human or louse strains of the Polish typhus fever virus.

DISCUSSION.

The experimental disease induced in guinea pigs with the human and louse strains of the Polish typhus virus is characterized by the following conditions: (*a*) Transmissibility of the virus from patient to guinea pig and from guinea pig to guinea pig indefinitely. (*b*) Specific pathology of the affected animals during the height of the reaction. The macroscopic examination reveals no changes in the organs with the exception of enlargement of the spleen[15] and a petechial rash in the deeper layers of the skin. The lesions in the different organs are demonstrable by histological study and consist of a particular vascular change, especially about the blood vessels of the brain. (*c*) Absence of concomitant or secondary infections by ordinary bacteria (pneumonia, peritonitis, abscesses, pseudotuberculosis, or other diseases, or the presence of these bacteria in the blood). (*d*) Specific immunity reactions. A guinea pig after reacting to an

[15] It has been stated that the organs in man (Nicol[4]) and in the guinea pig(Doerr[5]) show no macroscopic lesions whatever. But that the spleen is definitely enlarged has been our experience and this is confirmed by the following reports, both in regard to typhus fever in man and experimental typhus fever in animals: Ricketts, H. T., and Wilder, R. M., *J. Am. Med. Assn.*, 1910, liv, 463; Curschmann, H., Typhoid and typhus fever, Philadelphia, 1902, 526; Plotz, H., Olitsky, P. K., and Baehr, G., *J. Infect. Dis.*, 1915, xvii, 1; Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127; Grzywo-Dabrowski, W., *Virchows Arch. path. Anat.*, 1918, ccxxv, 299; Danielopolu, D., Le typhus exanthematique, Bucharest, 1919, 222; Shattuck, G. C., Typhus fever with particular reference to the Serbian epidemic, Cambridge, 1920, 111, and others.

injection of the virus remains immune to a subsequent inoculation of the typhus fever virus.

It appears then that the blood from the immigrant could induce in guinea pigs a similar experimental disease, for, as will be noted in the above experiments, it is characterized by (*a*) transmissibility, (*b*) particular pathological effects, (*c*) absence of concomitant or secondary infections by ordinary bacteria, and (*d*) specific immunity reactions. From the experiments on immunity we infer that the strain of virus inducing the typhus fever occurring last year in Warsaw, Poland, in epidemic form, and that inducing the disease in immigrants this year at the Port of New York, are identical.

CONCLUSION.

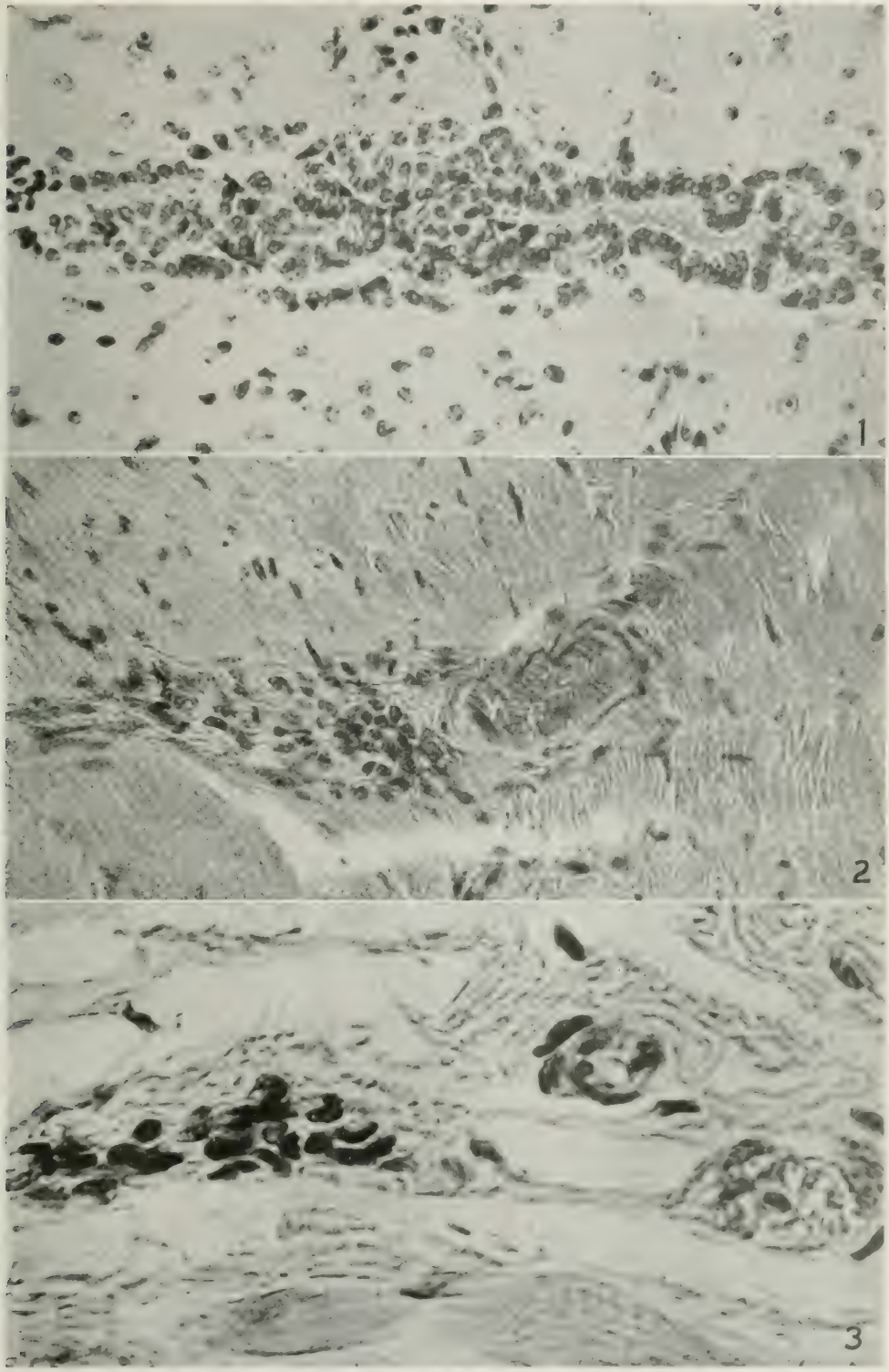
It is experimentally demonstrated in the guinea pig that the blood from an infected Czecho-Slovakian arriving at the Port of New York from Italy contained the virus of typhus fever and that this strain is identical with the epidemic virus present in man and louse in Poland.

EXPLANATION OF PLATE 25.

FIG. 1. Section of brain of guinea pig experimentally infected with the blood from the immigrant. The vascular lesions are shown in detail. There are circumscribed proliferation and accumulation of macrophages and leucocytes about an arteriole, and at one point, a *periarteriolitis nodosa*. × 400.

FIG. 2. Section of heart muscle from the same guinea pig. The vascular lesions are shown in detail. They resemble those found in the brain and demonstrated in Fig. 1. × 400.

FIG. 3. Section of the corium of the skin from a guinea pig experimentally infected with the blood from the immigrant (fifth guinea pig passage). The circumscribed accumulation of macrophages and leucocytes close to vessels, which show proliferation and swelling of the endothelial cells, is noteworthy. A thrombus is seen in one of the vessels. × 1,000.



(Olitsky: Typhus fever among recent immigrants.)

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STUDIES ON THE PHENOMENON OF D'HÉRELLE WITH BACILLUS DYSENTERIÆ.

By MARTHA WOLLSTEIN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 35 to 39.

(Received for publication, May 31, 1921.)

The phenomenon of d'Hérelle (1) is the expression of a lytic reaction occurring between a bacterium which is inducing an infection in an animal and a substance elaborated in that organism, probably by the leucocytes and other tissue cells, in response to the stimulus of the metabolic products of the invading bacterium. The important element of the reaction is that bacteria exposed to the lytic substance acquire the ability to transfer the lytic property to subsequent generations. The first observations of d'Hérelle were made on stool filtrates from patients convalescent from bacillary dysentery (1). As a result of this and further studies, d'Hérelle (2) concluded that whenever an animal offers resistance to a pathogenic bacterium, a bacteriophage, active for that bacterium, can be isolated from the dejections of the animal. He believes that defense by bacteriophage is a specific immunity reaction. Dumas (3) was able to demonstrate that filtrates from specimens of Paris city water, of Seine water, and of earth, were lytic for colon and dysentery bacilli. He consequently expressed the opinion that the phenomenon is non-specific. The explanation of Dumas' results, as McLeod (4) has stated, is that the specimens he examined were contaminated with excreta infected with colon or dysentery bacilli or both. Specimens from other places may give negative results.

EXPERIMENTAL.

Intestinal Contents.

In an attempt to demonstrate d'Hérelle's phenomenon in human intestinal contents, twenty-three patients at the Babies' Hospital were examined. Six of the children gave a history of gastrointestinal disturbance, while one had been observed throughout an attack of Shiga bacillus dysentery during which the bacillus had been isolated from the stools. Specimens from twenty-two of the twenty-three patients, including the dysentery convalescent, when grown in broth for 24 hours and then filtered through a Berkefeld candle, showed only a negative (non-lytic) reaction between the sterile filtrate and young cultures of *Bacillus dysenteriæ*. One positive reaction was obtained with the filtrate from the feces of an infant who had died of peritonitis due to *Bacillus coli* after intussusception of 3 days duration. In this instance the lytic action was stronger on the colon bacillus than on the dysentery bacillus, and it was possible to transfer the lytic quality to later generations of *Bacillus coli*.

The negative results obtained in the case of the infant who had recovered from an attack of Shiga dysentery may mean that the lytic quality of the intestinal contents is of short duration after such an infection. That the phenomenon of d'Hérelle is not a widespread one among children not ill of gastrointestinal disease is quite evidently demonstrated by these observations.

Dumas' (3) positive lytic reaction with the stools of five of eight adults who gave no history of previous intestinal infection would seem to show that there may be a difference between children and adults in this respect.

Guinea Pig Peritoneal Exudate.

Bordet (5) interpreted the lytic phenomenon of d'Hérelle as due to leucocytic action. He demonstrated its presence in the peritoneal exudate of guinea pigs inoculated with *Bacillus coli* on three occasions at intervals of 5 days. Repeated attempts to reproduce Bordet's results were negative, until a guinea pig was killed after only one intraperitoneal injection; that is, before the peritoneal exudate of the animal had become antilytic by repeated inoculations (Bordet).

The peritoneal exudate is withdrawn by means of sterile capillary tubes from the living animal, and diluted with twice its quantity of sterile broth adjusted to a pH of 8. This is in accordance with Gratia's (6) observation that the lytic action of d'Hérelle proceeds more readily and completely in an alkaline than in an acid medium. In order to rid the guinea pig exudate of fibrin it is well to draw it into a tube containing small sterile beads. The tube containing the defibrinated exudate is sealed and kept for 3 to 30 days to complete the leucocytic action (Bordet). At the end of that time it is centrifuged and the clear supernatant fluid heated for ½ hour at 58°C., after which it is ready to be tested on broth or agar slant cultures. Both must be young, not over 2 to 3 hours old. 1 drop of the fluid to be tested is dropped along one side of an inoculated agar slope, and the tube set upright in the thermostat for observation during the next 48 hours. To broth cultures a larger quantity of the fluid must at first be added, but after one or two generations the lytic fluid may be found to be active in high dilutions.

Growth may be prevented in a broth culture if the lytic agent be added when the tube is inoculated, or young bacilli may be dissolved if the fluid be added after the culture has grown 2 to 4 hours. A broth culture to which the lytic fluid has been added remains or becomes more or less clear for 12 to 24 hours, then it may become somewhat turbid, and clear again after another day. Even the clear tubes are not always sterile, as can be shown by plating. On agar slants the action of the lytic fluid is evidenced by a clear streak along the track of the fluid (Fig. 1). In this clear area there may be no colonies, or there may be a few; that is, some resistant bacilli may remain undissolved and viable. The action of the lytic fluid serves to separate a given culture into sensitive and resistant strains, and by repeating the action on the resistant strains a more and most resistant one can be obtained.

By filtering the clear fluid resulting from the action of the original lytic filtrate or exudate on a broth culture, any quantity of lytic fluid can be obtained, and its action on any number and variety of organisms tested.

The bacteriophage obtained by inoculating a strain of Shiga dysentery bacilli into a guinea pig was found to dissolve its homologous strain, five other Shiga bacillus strains, and two Flexner dysentery bacillus strains. On three strains of Hiss Y bacilli, however, its action was less marked; while three strains of colon bacilli, and one strain

each of typhoid, paratyphoid A and B, mouse typhoid, Morgan bacillus, hog-cholera, and fowl-cholera were not attacked.

Dr. Gratia kindly gave me a small quantity of a lytic fluid which he had prepared by allowing guinea pig exudate, obtained by Professor Bordet in Brussels, to act on a resistant strain of *Bacillus coli*. This bacteriophage proved to be strongly lytic for strains of *Bacillus dysenteriæ* Shiga and less lytic for two strains of Flexner and three strains of Hiss Y dysentery bacilli. The Brussels strain of *Bacillus coli* as well as a strain isolated at the Babies' Hospital some months ago were readily dissolved, but a recently isolated strain of colon bacilli was not affected. *Bacillus typhosus* was acted upon to a slight extent only, but neither a paratyphoid A nor B strain was attacked.

These results showed that the lytic power of the colon phage, made with a resistant *Bacillus coli* strain, is not specific, but that it acts in varying degree on the related strains of the colon-typhoid-dysentery groups of bacilli. The observations of Bordet and Ciuca (7) announcing this fact were published just as our experiments were completed.

One strain each of Friedländer's bacillus, *Bacillus choleræ suis*, *Bacillus avisepticus*, *Bacillus typhi murium*, and Morgan's bacillus was tested with negative results.

With the purpose of studying more fully the effect of the action of a bacteriophage on the dysentery bacillus, a strain of the Shiga type was selected and exposed to the action of the colon phage. A new bacteriophage resulted which was strongly lytic for all strains of dysentery bacilli, for typhoid bacilli, and for the Belgian and American strains of *Bacillus coli*. The paratyphoid strains were still unaffected.

The anti-colon bacillus bacteriophage showed certain group reactions on members of the dysentery-colon-typhoid group. It was most active on the colon strain with which it had been made, less active on a heterologous colon strain; equally active on Shiga dysentery strains, less so on Flexner strains, and still less so on Hiss Y strains; and it was only slightly active on a strain of typhoid bacilli. The Shiga antidysentery bacillus phage, on the other hand, was completely lytic on all strains of Shiga dysentery bacilli and only slightly less so on all strains of Flexner and Hiss Y strains. It was completely lytic for Belgian and American strains of *Bacillus coli* and decidedly lytic for a typhoid bacillus strain. Its group reactions

were stronger than those of the colon bacillus phage. The question naturally presented itself as to whether this denotes a stronger bond between typhoid and dysentery bacilli than between dysentery and colon bacilli, or whether the lytic power of the dysentery bacteriophage was fundamentally stronger for all strains of these three groups. Weight is given to the former supposition by the fact that while the colon phage acted very slightly on the typhoid bacillus, a typhoid phage made from the colon phage by exposure to a strain of *Bacillus typhosus* acted much more strongly on dysentery than on *coli* strains.

Further study of the effect of the action of a lytic fluid upon a strain of Shiga dysentery bacilli is of interest. Not all the bacilli are dissolved, and it follows that those which survive are resistant to the lytic principle. Plates poured from a fluid culture of Shiga dysentery bacilli after 24 or 48 hours contact with a lytic fluid, when the broth culture tube appears to be perfectly clear, show two kinds of colonies (Figs. 2 to 4). One is round, with regular edges, a heaped up or denser center, and rather moist growth. The second kind is very irregular in size and shape, with deep indentations in its thin edges, a depressed center, and very granular. In broth the regular colonies grow rapidly and profusely, the broth becoming turbid. The irregular colonies grow more slowly and fall to the bottom of the tube in granular masses, leaving the broth slightly turbid or almost clear.

A strain of Flexner dysentery bacilli exposed to dysentery bacillus bacteriophage gave rise to regular and irregular colonies exactly comparable to those obtained from the acid or Shiga dysentery strains. Kuttner (8) has shown that the typhoid bacillus develops two kinds of colonies after action of a typhoid bacteriophage.

The experiment presented in Table I was done in order to test the reaction of the bacilli in the two kinds of colonies to the lytic fluid. The table shows that the bacilli in the regular colonies are more resistant to the lytic principle than are those in the irregular colonies.

To ascertain which colonies carry the lytic agent, *i.e.* which are lysogenic, the experiment presented in Table II was done. The irregular colonies are found, then, to be lysogenic and sensitive, while the regular colonies are non-lysogenic and resistant.

Both the regular and the irregular colonies were subcultured on plates for more than 40 generations, and Figs. 5 and 6 show the results. The regular colonies retain their regular outline and characteristics. The irregular colonies, on the other hand, gradually lose their markedly irregular outline and tend to approximate more nearly the regular type of colony, until there are more regular than irregular ones present, and even these are less radically irregular. That is, while the edges of the irregular colonies may still be deeply serrated, the centers are

TABLE I.

Shiga Dysentery Bacilli Plus Shiga Bacteriophage.

Strain.	Medium.	Age.	Result.	
			After 24 hrs.	After 48 hrs.
		hrs.		
Normal, or original	Broth.	2	Lysis; tube clear.	Same.
Regular	"	2	No lysis; tube turbid.	"
Irregular	"	2	Lysis; tube clear.	"

TABLE II.

Normal or Original Strain of Shiga Dysentery Bacillus.

Phage.	Medium.	Age.	Result.
		hrs.	
Normal, original bacillus filtrate	Broth.	2	Lysis.
Regular colony filtrate	"	2	No lysis.
Irregular " "	"	2	Complete lysis.

less hollowed out and gradually become dense (Figs. 7 and 8). The explanation seems to be that the sensitive bacilli die off more rapidly than the resistant ones, which form the regular colonies in later generations. It is a matter of selection.

The two kinds of dysentery bacilli, resistant and sensitive, do not differ from the original culture in their sugar reactions.

An attempt was made to determine whether the acquired lysogenic property is retained permanently or lost in later generations. Working with the original, normal culture as a whole, it was found that

after the seventh generation on agar or in broth the bacilli which had survived contact with a lytic fluid were no longer able to transmit the lysogenic property to other cultures or to dissolve the normal Shiga bacilli. Apparently the sensitive, lysogenic bacilli had all been eliminated in seven generations.

The experiments on this point were repeated with bacilli from colonies isolated from a normal Shiga culture which had been exposed to dysentery bacteriophage and then plated. The resulting, undissolved bacilli must all be resistant to some degree, but the colonies which grew were of the two distinct types noted above, and were designated sensitive resistant, or irregular, and resistant resistant, or regular. The bacilli from the regular colonies were not dissolved by the dysentery bacteriophage, nor did their broth filtrate dissolve normal Shiga bacilli of the original strain, or the bacilli of the irregular colonies. On bacilli from the regular colonies some lysis resulted. The bacilli from the irregular colonies, on the other hand, were dissolved by the dysentery phage and by their homologous filtrate, which also dissolved normal dysentery bacilli and acted to some extent on the bacilli of the regular type of colony. This was explained by the assumption that the culture of regular, resistant bacilli was not absolutely pure in the sense that some less resistant bacilli were still present. By plating, this was proved to be correct, for both regular and irregular colonies developed from a broth tube of regular bacilli which had been in contact with lytic fluid over night. The regular colonies fished from this plate were again subjected to the action of the strong phage, and plated after 4 days. The resulting colonies were all of the regular type, and were not dissolved by the strong dysentery phage or by filtrates from regular or irregular types of bacilli. It is possible then to obtain a pure, resistant type of dysentery bacilli by repeated elimination of sensitive bacilli from the strain, these resistant bacilli having been present originally.

The sensitive type of dysentery bacillus, after nine generations of broth and plate subculturing, was found to have become more resistant, as shown by the fact that the bacilli of the ninth generation were acted upon less strongly by the antidysenteric phage and by the filtrate from the first generation of irregular bacilli. The filtrate

from the ninth generation was also less lysogenic than the filtrate from the first generation had been.

The sensitive bacilli gradually die out by the action of the lytic substance they carry, and gradually resistant bacilli only are left. The tendency then is for cultures of sensitive bacilli to become more resistant and for resistant bacilli to remain resistant. This is also demonstrated by the colonies on repeated subcultures (Figs. 5 and 6).

The Lytic Reaction under Anaerobic Conditions.

The lytic action proceeds as rapidly and as completely in the absence of oxygen as in its presence. Deep broth cultures with rabbit kidney and sealed with vaseline were inoculated with dysentery bacilli and antidysenteric phage, and growth was prevented as it was in the aerobic controls.

Morphology.

The sensitive strains are composed of short bacilli and many coccoid and round, swollen forms. The resistant strains are made up of regular, equal sized, and evenly staining bacilli, with some larger forms, but few, if any, coccoid or swollen, round forms. Threads are seen in older cultures of the more resistant types.

Virulence.

Rabbits inoculated with minute doses of early broth cultures of the resistant bacilli became paralyzed within 48 hours, and did not recover. Cultures of the sensitive bacilli required five times as great a dose to produce similar results. Apparently the exotoxin of the resistant type of dysentery bacilli is much stronger than that of the sensitive type.

Immunization.

Rabbits were immunized with resistant strains of Shiga dysentery bacilli, with normal strains, and with Shiga bacteriophage. Agglutination tests with the resulting sera brought out the fact that normal, irregular or sensitive, and regular or resistant strains are agglutinated well, the resistant somewhat less so than the sensitive. But a highly resistant strain was not agglutinated even by the sera made with

ordinarily resistant strains. The highly resistant strain killed the animals before they developed immune bodies in their sera.

The animals immunized with antidysenteric phage agglutinated normal dysentery strains in dilution of 1:200, sensitive strains in 1: 1,000, and resistant strains in 1: 100, while the highly resistant strain was not agglutinated.

Evidence which shows that d'Hérelle's phenomenon goes on in the human intestine in a patient with dysentery was recently obtained. At autopsy on an infant 9 months old, who died of bronchopneumonia, a severe pseudomembranous enterocolitis was found. The child had only been under observation for 48 hours, but a history of intestinal symptoms was obtained. By proctoscopic examination on the day before death ulcers were seen in the rectum and a specimen of feces was obtained and plated. On the plates Flexner dysentery bacillus colonies developed with marked, irregular, indented edges. A few small, regular colonies were also noted. Fishings from both kinds of colonies were made, and a sensitive and a resistant strain was obtained. The plates made directly from the rectal specimen were very similar to those obtained in our experiments on the action of the antidysentery lytic fluid on dysentery bacilli. Whether, if a complicating pneumonia had not intervened, the child would have recovered from the dysentery infection or whether the resistant dysentery bacilli were present in sufficient numbers to prevent recovery in spite of the active lysis going on, remains a question.

SUMMARY.

It has been shown that a lytic fluid for dysentery bacilli can be obtained from the peritoneum of the guinea pig by intraperitoneal inoculation of live dysentery bacilli, and that the lytic action of such a fluid is not strictly specific, but that it exerts a group action on the dysentery-colon-typhoid group of bacilli. A lytic fluid with similar effects was obtained from a child dying of Flexner dysentery infection, and an anti-colon bacillus lytic fluid from a child who died of intussusception with colon bacillus peritonitis.

The action of the lytic fluid on the dysentery bacilli, both *in vivo* and *in vitro*, is to divide the culture into sensitive and resistant strains, and the latter can be carried to a degree of very marked, if not complete resistance to lysis. Such resistant strains are not lysogenic, nor are

they agglutinable. The sensitive strains are lysogenic and agglutinable. Varying degrees of sensitive and resistant bacilli exist in a single culture. The sensitive bacilli gradually lose the lysogenic property which they acquired under special conditions, but the very resistant bacilli never acquire that property. It is conceivable that the resistant strains are responsible for the untoward outcome of disease in human beings. Theoretically the administration of lytic fluid should rid the intestinal tract of most of the infecting bacilli, and only if completely resistant bacilli in large numbers remain unacted on is the outcome of the disease a fatal one.

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EXPLANATION OF PLATES.

Plate 35.

Fig. 1. Effect of the bacteriophage on an agar slope culture of *B. dysenteriæ*.

Fig. 2. Agar plate of a culture of *B. dysenteriæ*.

Plate 36.

Fig. 3. Regular colonies of *B. dysenteriæ* after the action of the bacteriophage on a normal strain.

Fig. 4. Irregular colonies of *B. dysenteriæ* after the action of the bacteriophage on a normal strain.

Plate 37.

Fig. 5. Regular colonies of *B. dysenteriæ* after 40 generations of growth on agar plates.

Fig. 6. Irregular colonies of *B. dysenteriæ* after 40 generations of growth on agar plates.

Plate 38.

Fig. 7. Irregular colonies of *B. dysenteriæ;* third generation; thin edges and hollowed centers.

Plate 39

Fig. 8. (*a*) Regular colonies of *B. dysenteriæ;* 40th generation. (*b*) Irregular colonies; 40th generation; irregular edge and heaped up center.

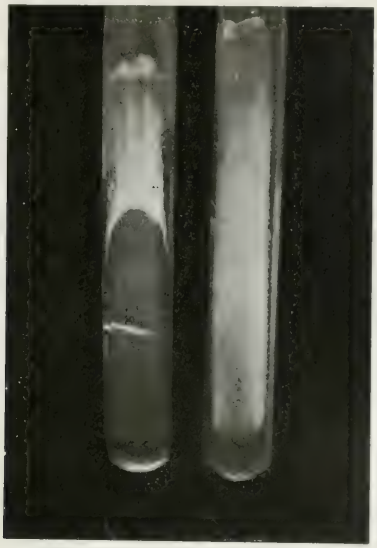

FIG. 1.

FIG. 2.

(Wollstein: Phenomenon of d'Hérelle.)

FIG. 4.

FIG. 3.

(Wollstein: Phenomenon of d'Hérelle.)

FIG. 6.

FIG. 5.

FIG. 7.

(Wollstein: Phenomenon of d'Hérelle.)

FIG. 8.

(Wollstein: Phenomenon of d'Hérelle.)

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STUDIES ON THE TREATMENT OF HUMAN TRYPANOSOMIASIS WITH TRYPARSAMIDE (THE SODIUM SALT OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID).

By LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

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INTRODUCTION.

The work in experimental chemotherapy of certain protozoan infections which has been in progress at the Rockefeller Institute for several years, has resulted in the development of a large series of compounds, of which one at least was found to possess a high degree of therapeutic activity in experimental trypanosomiasis. This substance, *N*-phenylglycineamide-*p*-arsonic acid, was first made in 1915 by Dr. WalterA. Jacobs and Dr. Michael Heidelberger (1 and 2) and studied biologically in collaboration with Dr. Wade H. Brown under its serial number of A 63; its sodium salt has recently been named "Tryparsamide." The biological investigations included toxicologic experiments (3) in which it was shown that the reaction of different species of laboratory animals to the drug was of a favorable character. The substance lends itself well to almost any method of administration and can be given to animals in large doses. Moreover, toxic effects were confined to doses relatively close to the minimum lethal dose and the recovery of animals from sublethal intoxication was remarkably rapid and complete, thus making possible the repetition of large doses at comparatively short intervals of time. The therapeutic activity of the drug in experimental trypanosomiasis (4) was particularly evidenced by the relative speed and sharpness of action in the acute blood infections of mice and rats and by the potency and duration of action in the subacute and chronic tissue infections of guinea pigs and rabbits. The accomplishment of a permanent cure was obtained in the experimental infections produced by five strains of pathogenic trypanosomes, *Tr. brucei*, *Tr. gambiense*, *Tr. evansi*, *Tr. equiperdum*, and *Tr. equinum*, but, on the other hand, infections produced by *Tr. rhodesiense* were more difficult to influence (5). Comparative experiments in laboratory animals with well known drugs such as atoxyl, arsacetin, arsenophenylglycin, salvarsan, neosalvarsan, luargol, and galyl, all of which have been used in the treatment of human trypanosomiasis, showed that tryparsamide was in many respects their superior.

An appraisal of the probable value of any drug, with a view to its use in the treatment of human beings or animals suffering from naturally acquired trypanosomal infections, must be based upon the interpretation of the experimental results obtained with the drug in

the laboratory. The various attributes or features of the toxicologic and therapeutic action of tryparsamide, as brought out by laboratory investigation and briefly enumerated above, constituted a logical basis for its application to the treatment of human and animal trypanosomiasis. This conclusion is emphasized by recalling the results of the use of several drugs in human trypanosomiasis, for which various claims had been made on the basis of limited or mistakenly interpreted experimental data. For instance, arsacetin, arsenophenylglycin, salvarsan, and neosalvarsan all possess a high curative ratio in experimental trypanosomiasis of mice and largely upon this basis were transferred to the field of human therapy and that of the large domestic animals. The regrettable toxic effects sometimes noted and the failure to obtain the desired therapeutic action under the conditions imposed by the disease, might have been foretold if requisite laboratory experiments had been carried out and correctly interpreted. A similar statement may be made of so called trypanocidal tests made *in vitro*. On the other hand, the better therapeutic results obtained with atoxyl are entirely in accord with the results of the laboratory experiments carried out by Thomas and Breinl (6 and 7) and aptly illustrate the point in question.

At the present time, human trypanosomiasis, or the so called African sleeping sickness, is very prevalent in tropical Africa, particularly in the central and western portions of the continent, in the French and Belgian Congos. Epidemics of varying degrees of severity are widespread and constitute one of the most pressing problems confronting colonial governments. Although great progress has been made in the fight against the disease and notably so in the treatment of patients in the early stages of the infection by the use of atoxyl or by the combined treatment with atoxyl and tartar emetic, it is apparent that the means of combating the disease, particularly in its more advanced stages and in prophylaxis against its spread, are not at present adequate. Whether the ideal control of an infection of the nature of trypanosomiasis will ever be accomplished by a single drug, in the sense that quinine is used to control malaria, is problematical. In any event, the various aspects of the situation in Africa today are such that the colonial officials are very ready to try any new remedy, itself harmless, for the treatment of sleeping sickness which holds out

a promise of some degree of success. The biological experiments referred to indicated that tryparsamide might fill some place in the therapy of the disease and consequently a mission was sent by the Rockefeller Institute to the Belgian Congo in May, 1920, to study the effect of the drug in patients suffering with trypanosomiasis.

It is obvious that any extended application of a new remedy for administration in human beings must be preceded by a careful study of its action in relatively few patients irrespective of the scope of the initial investigations in the laboratory. In a problem such as the therapy of human trypanosomiasis as just indicated, it is of the utmost importance to ascertain such fundamental facts as the initial speed of therapeutic activity of various sized single doses, as measured by the length of time required to clear the superficial lymph glands and blood of trypanosomes and the permanency of this effect; the action of the drug, if any, in the advanced stages of the disease as indicated by examination of the spinal fluid and the mental and nervous response of the patient; the general physical reaction of the patient as shown by weight observations, blood counts, etc.; and finally, the determination of any untoward observable effects. On the basis of a series of such facts obtained in a certain number of patients, a rational system of therapeutic procedure can then be logically attempted.

The object of the mission sent to the Belgian Congo was the obtaining of concrete facts and observations as outlined above. All of the work was done at the government laboratory and Hôpital de la Reine in Léopoldville. The helpful cooperation of the Belgian and Colonial Governments made it possible to accomplish a considerable amount of work in a comparatively short time and it is a pleasure to express here our appreciation of the aid so cordially extended to us, first in Brussels and then in the Congo. We also wish to acknowledge the assistance given us by Professor Rodhain, Chief of the Colonial Medical Service, and by Dr. van den Branden, Director, and Dr. van Hoof, Assistant Director of the Laboratory in Léopoldville.

SOURCE AND TYPE OF PATIENTS.

The observations which form the basis of this report were made on 77 native cases of trypanosomiasis caused by *Tr. gambiense* which were treated with tryparsamide in Léopoldville, Belgian Congo. The

civil service of the Belgian Congo has established throughout the colony, medical stations and posts under the charge of government physicians and traveling sanitary agents, in addition to which a considerable amount of medical work is done by various religious missions. The native population is thus examined for trypanosomiasis at more or less regular intervals, the routine procedure consisting of the palpation of the cervical lymph glands and the microscopic examination of lymph gland juice from those individuals having one or more palpable cervical glands. At the present time, there is no widespread epidemic of a severe character in the immediate vicinity of Léopoldville but there are many endemic foci of the disease in this district, while a few days' travel brings one into active epidemic areas. The patients treated with tryparsamide were entirely typical of the routine run of trypanosomiasis cases met with in Léopoldville. The population of the nearby native villages comes *en masse* to the laboratory every 3 months for examination and several cases were obtained in this way. Moreover, Léopoldville is the terminus of one of the large river transportation companies and a medical passport issued after examination for trypanosomiasis, is required of any native traveling from one part of the colony to another. The entire crew of each boat is examined the day before sailing and this source yielded a number of cases from distant parts of the colony. Finally, several advanced cases under treatment in the native Hôpital de la Reine and lazaret in Léopoldville were transferred to us for treatment with tryparsamide. All of the patients studied thus fell into three general classes: First, those who were sent to the lazaret, which is in reality a native village under nominal native police control on the outskirts of Léopoldville, and who came to the laboratory when sent for; second, patients in the Hôpital de la Reine, over whom a closer supervision could be maintained; and third, ambulatory cases, corresponding to dispensary patients, who continued to live in their own villages and who came to the laboratory at certain fixed times.

According to the recognized authorities on human trypanosomiasis, the course of the disease may be divided into two periods, the glandular or blood infection stage and the cerebral stage. The first stage is characterized principally by a polyadenitis, most apparent in the

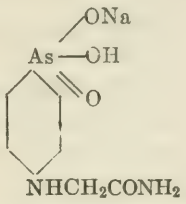
cervical glands, an irregular intermittent fever, a rapid pulse (the rate of which is usually out of proportion to the fever), an enlarged spleen, an erythematous eruption in whites, a fine tremor of the tongue, a deep hyperesthesia, and asthenia. Localized edemas are sometimes seen. Trypanosomes are present in the hypertrophied lymph glands and in the blood. The number of lymphocytes in the spinal fluid is normal. The second period, in which the infection has invaded the cerebrospinal system, is characterized by the aggravation of the general symptoms such as fever, emaciation, loss of strength, and by the predominance of cerebrospinal symptoms such as tremors, paralysis, mental disturbances, apathy, somnolence, lethargy, and finally coma and death. In this period, the spinal fluid is pathologically altered, the conspicuous feature of which is an increase in the lymphocytic (small mononuclear) cell content with other types of cells (large mononuclears and so called mulberry cells) in the more advanced cases (8 and 9). Trypanosomes are present in the spinal fluid of the most advanced cases while they are said to be less numerous in the lymph glands and blood than in the early cases.

The 77 cases treated with tryparsamide represented all grades of the infection and included previously untreated patients as well as several who had been treated with one or more drugs for varying lengths of time. With the exception of 8 patients who were being treated at the time of their transfer to us, trypanosomes were demonstrated in every case as follows: in 67 cases by gland punctures; in 1 case in the centrifuged blood; in 1 case in the spinal fluid. A lumbar puncture was done before treatment in 72 instances—it was omitted in the case of 1 infant and 4 ambulatory patients.

The study of tryparsamide was carried out under the same conditions that prevail in the laboratory, lazaret, and native hospital in Léopoldville, under which the routine treatment with atoxyl and tartar emetic is given. Under the conditions prevailing, complete clinical records were not obtainable and the observations were necessarily confined to certain definite fields. In addition, mention should be made of the fact that the native population of West Africa suffers widely from other affections such as subacute and chronic malaria, filariasis, and intestinal parasites, of which little or no attention could be taken.

ADMINISTRATION OF TRYPARSAMIDE.

Tryparsamide, or the sodium salt of *N*-phenylglycineamide-*p*-arsonic acid, is a pentavalent arsenic compound having the following structural formula:



Its preparation and chemical properties have been fully described by Jacobs and Heidelberger in previous publications (1 and 2). It is a white crystalline substance of stable character and has been kept in ordinary tightly stoppered bottles for long periods of time and under various climatic conditions without appreciable change. It was found that the same procedures of preparation of drug solution employed in the experimental work (3 and 4) were entirely applicable to the preparation of the drug for human therapy.

As the drug is bottled under aseptic conditions, the addition of sterile distilled water with the usual precautions for preventing contamination is all that is required. Sterilization of the drug solution is not necessary. The dose for each patient was in most instances weighed out separately on a balance and dissolved in a small sterile petri dish or flask with the appropriate amount of sterile distilled water. It is entirely feasible, however, to prepare the doses for several patients at one time and to administer the requisite amount of the drug to each. The drug is extremely soluble in water in concentrations as high as 50 per cent. In most instances, a 20 per cent solution was used, but varying strengths, from 10 to 40 per cent, were also employed.

The drug is readily administered intravenously and this route obviously offers the most uniform condition for the observation of various sized doses. When injected directly into the tissues, it is quickly absorbed and produces but slight irritation or local injury. However, the African native exhibits little objection to intravenous

medication while opposing injection into the subcutaneous or intramuscular tissues. In a few patients injections deeply into the muscles of the buttocks could be made and the effects studied.

ADMINISTRATION OF SINGLE DOSES IN EARLY CASES.

Initial Peripheral Sterilization.

The characteristics of human trypanosomiasis would seem to preclude any idea of materially influencing the course of the disease by the administration of a single dose of any drug and the experience of tropical workers indicates that a series of treatments covering a period of months or years is necessary. Untreated cases eventually die and it is the general opinion that only comparatively slight and temporary benefit accrues to patients in the advanced or cerebrospinal stage of the disease from the drugs hitherto in use. Before any system of treatment can be intelligently attempted, it is necessary to determine the duration of therapeutic action of single doses since the

TABLE I.

Results of Early Gland Puncture After Single Doses of Tryparsamide.

Number of patients—55.
Size of doses—0.3 to 7.0 gm. or 4.7 to 112 mg. per kilo of body weight.
Routes of administration—Intravenous 51.
Intramuscular 4.

Results:

All examinations negative within 24 hours after treatment.
20 " " " 4½ to 9 hours after treatment.
10 " " " 10 to 15 " " "
13 " " " 15½ to 20 " " "
12 " " " 21 to 24 " " "

Time of negative examination after treatment.	Doses of 0.3 to 1.0 gm. 4.7 to 25 mg. per kg.	Doses of 1.0 to 3.0 gm. 33 to 50 mg. per kg.	Doses of 2.0 to 5.0 gm. 54 to 70 mg. per kg.	Doses of 4.0 to 7.0 gm. 80 to 112 mg. per kg.
I. 4½ to 9 hours.....		10	4	6
II. 10 to 15 "	3	3	3	1
III. 15½ to 20 "	4	3	3	3
IV. 21 to 24 "	4	4	2	2
Total.....	11	20	12	12

giving of succeeding doses should be governed by this factor. In addition, this information is of importance in the prophylactic use of a drug. Incidentally, it was desirable to ascertain the approximate rapidity of the preliminary or so called sterilizing effect of a given dose as shown by repeated microscopic examinations of lymph gland juice or of centrifuged blood. A number of such observations were made, which are summarized in Tables I and II. The patients represent both early and advanced periods of the disease in which trypanosomes were demonstrated before treatment in the lymph glands or blood.

The gland punctures of 55 patients were all negative within 24 hours after treatment with single doses of tryparsamide varying from 0.3 to 7.0 gm. or 4.7 to 112 mg. per kilo of body weight; 30 examinations were negative within the first 15 hours, 25 from 15 to 24 hours after treatment, irrespective of the size of the dose (Table I). It should be pointed out that these results do not imply that an examination one or more hours previous to the negative finding was positive, as it was not always possible to make a complete series of examinations at frequent intervals since microscopic work could only be done in the daytime and consequently a relative and not absolute interpretation must be placed upon the observations. In 14 cases, trypanosomes were found microscopically in lymph gland juice 3 to 8 hours after doses of 1.0 to 5.0 gm., while the examinations repeated within the succeeding 21 to 16 hours were invariably negative. The following examples are typical of the results obtained:

Makalamba № 49. +. Tryparsamide 1.0 gm. or 19 mg. per kilo.
Gland puncture: 6 hours +; 19½ hours 0.

Mondeke № 47. +. Tryparsamide 2.0 gm. or 33 mg. per kilo.
Gland puncture: 5½ hours +; 6 hours 0.

Masimango № 16. ++. Tryparsamide 2.0 gm. or 42 mg. per kilo.
Gland puncture: 6 hours +; 20 hours 0.

Tono № 14. ++. Tryparsamide 2.0 gm. or 44 mg. per kilo.
Gland puncture: 8 hours +; 9 hours 0.

Loyoko № 18. ++. Tryparsamide 3.0 gm. or 50 mg. per kilo.
Gland puncture: 5 hours +; 8 hours 0.

Kaniki № 58. +++. Tryparsamide 2.5 gm. or 58 mg. per kilo.
Gland puncture: 5 hours +; 21 hours 0.

Tuamene ✻68. ++. Tryparsamide 5.0 gm. or 82 mg. per kilo.
Gland puncture: 3 hours +; 8 hours 0.
N'Siola ✻32. +. Tryparsamide 4.0 gm. or 100 mg. per kilo.
Gland puncture: 4 hours +; 6 hours 0.

In contrast to the above examples, the following 3 cases are of interest in that gland punctures were positive 14 and 19 hours after treatment. The doses used however, in terms of amounts of drug per kilo of body weight were comparatively small and the infection of 2 patients was unusually severe.

Bikoko ✻55. +++. Tryparsamide 0.3 gm. or 4.7 mg. per kilo.
Gland puncture: 19 hours +; 22 hours 0.
Molalo ✻60. +. Tryparsamide 1.0 gm. or 20 mg. per kilo.
Gland puncture: 14 hours +; 16 hours 0.
Tsarjembe ✻74. +++. Tryparsamide 2.0 gm. or 37 mg. per kilo.
Gland puncture: 14 hours +; 15 hours 0.

Thus, it is seen that with amounts above 25 mg. per kilo, 27 of the examinations were negative within the first 15 hours, while 9 were negative in from $15\frac{1}{2}$ to 20 hours. 20 patients were treated with doses of 1.0 to 3.0 gm. or 33 to 50 mg. per kilo of body weight, of which 10 were negative within 9 hours, 3 within 10 to 15 hours, 3 within $15\frac{1}{2}$ to 20 hours, and 4 within 21 to 24 hours. Considering together the doses of from 33 to 70 mg. per kilo of body weight and combining the results of the second and third columns of Table I, it is seen that 14 of the 32 patients, or approximately one half, were negative within the first 9 hours, while there were 6 negative examinations each in the succeeding periods of time. Concerning smaller doses (4.7 to 25 mg. per kilo), all that can be said at present is that the examinations of the 11 patients so treated were all negative within 24 hours and there was practically no difference between the second, third, and fourth periods of time, *i. e.*, it is probable that all 11 were negative within 15 hours after treatment. With the larger doses of 80 to 112 mg. per kilo of body weight 6 of the 12 patients gave negative gland punctures within the first 9 hours after treatment. 4 patients were given doses of 1.0, 2.0, and 3.0 gm. intramuscularly with no delay in negative gland puncture.

The preliminary effect of the drug upon the blood infection may next be considered, since in reality so called peripheral sterilization includes both the superficial lymph glands and the circulating blood. The patients in this group represent both early and advanced periods of the disease of different degrees of severity. Doses of 1.0 to 5.0 gm. or 18 to 109 mg. per kilo of body weight were administered intravenously, and at periods ranging from 15 minutes to 30 hours afterwards 9 cc. of blood were withdrawn in a syringe containing 1 cc.

TABLE II.

Results of Early Blood Examination after Single Doses of Tryparsamide.

Number of patients—35.
Size of dose—1.0 to 5.0 gm. or 18 to 109 mg. per kilo of body weight.
Intravenous administration.

Results:

All negative within 21 hours after treatment.
14 " " 3½ to 9 hours after treatment.
13 " " 10 to 15 hours " "
8 " " 16 to 21 hours " "

Time of negative examination after treatment.	Doses of 1.0 gm. 18 to 25 mg. per kg.	Doses of 1.0 to 2.5 gm. 33 to 48 mg. per kg.	Doses of 3.0 to 5.0 gm. 51 to 70 mg. per kg.	Doses of 2.0 to 5.0 gm. 71 to 109 mg. per kg.
I. 3½ to 9 hours.....	1	5	2	6
II. 10 to 15 "	2	5	3	3
III. 16 to 21 "	3	1	4	
Total.....	6	11	9	9

of a 6 per cent sodium citrate solution and the mixture was then centrifuged three times according to the method described by Broden (10). The three sediments were examined microscopically for trypanosomes. The results of these examinations of 35 patients are condensed in Table II and may be described briefly: All the examinations were negative in 21 hours, 14 were negative in 3½ to 9 hours, 13 in 10 to 15 hours, and 8 in 16 to 21 hours, irrespective of the size of the dose administered. There were 11 patients treated with single doses of 1.0 to 2.5 gm. or 33 to 48 mg. per kilo of body weight and

5 of the 11 showed negative blood examinations within 9 hours after treatment, and 10 were negative within 15 hours. Combining the results of these doses with those of 51 to 70 mg. per kilo as shown in the second and third columns, 15 out of the 20 patients so treated were negative within 15 hours. Somewhat larger doses of 2.0 to 5.0 gm. or 71 to 109 mg. per kilo of body weight were given to 9 patients, all of whom were negative in 15 hours and 6 negative within 9 hours after treatment.

As in the microscopic examination of lymph gland juice, it was not possible to make complete series of observations. In these 35 patients, the last positive blood examinations occurred from 3 to 8 hours after treatment with doses of 1.0 to 3.0 gm. or 18 to 51 mg. per kilo. The following examples illustrate these points:

Molalo № 60. Tryparsamide 1.0 gm. or 20 mg. per kilo.
Centrifuged blood: 16 hours 0.
Mafuala № 30. Tryparsamide 1.0 gm. or 25 mg. per kilo.
Centrifuged blood: 6 hours 0.
Mago № 28. Tryparsamide 1.0 gm. or 33 mg. per kilo.
Centrifuged blood: 7½ hours 0.
Messamba № 57. Tryparsamide 2.0 gm. or 39 mg. per kilo.
Centrifuged blood: 7 hours +; 21 hours 0.
Mondi № 75. Tryparsamide 2.0 gm. or 44 mg. per kilo.
Centrifuged blood: 3½ hours 0.
Mulamba № 39. Tryparsamide 3.0 gm. or 51 mg. per kilo.
Centrifuged blood: 3 hours +; 8 hours 0.
Mondeke № 47. Tryparsamide 4.0 gm. or 66 mg. per kilo.
Centrifuged blood: 6½ hours 0.
Mambula № 43. Tryparsamide 2.0 gm. or 71 mg. per kilo.
Centrifuged blood: 6 hours +; 8 hours 0.
Bajaka № 41. Tryparsamide 4.0 gm. or 76 mg. per kilo.
Centrifuged blood: 15 hours 0.
Bikoko № 55. Tryparsamide 5.0 gm. or 80 mg. per kilo.
Centrifuged blood: 5 hours 0.

The negative gland and blood findings after treatment were controlled by subsequent examinations as indicated by the following examples:

Bikoko № 55.

Admitted to lazaret September 22, 1920. 64.5 kilos.
Cervical lymph glands markedly enlarged. Gland puncture +++.
Lumbar puncture normal.

1920												
Sept.	22,	5 p.m.	Tryparsamide 0.3 gm. (4.7 mg. per kg.) adm. i.v.									
"	23,	7 a.m.	14 hours after treatment			G. P.	+	(2 tryp.)				
		9 a.m.	16	"	"	"	"	"	+	(2 ")		
		12 n.	19	"	"	"	"	"	+	(1 ")		
		3 p.m.	22	"	"	"	"	"	0			
		5 p.m.	24	"	"	"					C.B.	0
"	24,	9 a.m.	40	"	"	"	"	"	0			
"	25,		3 days	"	"	"	"	"	0			
"	27,		5	"	"	"	"	"	+	(1 tryp.) " "		+
"	27,	11.30 a.m.	Tryparsamide 5.0 gm. (80 mg. per kilo) adm. i.v.									
		4.30 p.m.	5 hours after treatment								C.B.	0
		5	p.m.	5½	"	"	"	G. P.	0			
"	28,	7	a.m.	19½	"	"	"				" "	0
		10	a.m.	22½	"	"	"	"	"	0		
Oct.	2,		4 days	"	"	"	"	"	0			
"	4,		6	"	"	"	"	"	0		" "	0

Tsarjembe № 74.

Admitted for ambulatory treatment October 11, 1920. 53½ kilos.
Cervical lymph glands enlarged, typical chains. Gland puncture +++.
Centrifuged blood ++.
Lumbar puncture 19 lymphocytes per cmm. Large mononuclears ++.

1920												
Oct.	11,	5 p.m.	Tryparsamide 2.0 gm. (37 mg. per kg.) adm. i.v.									
"	12,	7 a.m.	14 hours after treatment			G. P.	+	(2 tryp.)				
		8 a.m.	15	"	"	"	"	"	0		C. B.	0
		9 a.m.	16	"	"	"	"	"	0			
		10 a.m.	17	"	"	"					" "	0
		11 a.m.	18	"	"	"	"	"	0			
"	13,		7 days after treatment				"	"	0		" "	0

Mondongo № 5.

Admitted to lazaret July 14, 1920. 47 kilos.
Cervical lymph glands enlarged. Gland puncture +.
Lumbar puncture 7.5 lymphocytes per cmm.

1920												
July	14,	10 a.m.	Tryparsamide 2.0 gm. (43 mg. per kg.) adm. i.v.									
		11 a.m.	1 hour	after	treatment		G. P.	+				
		12 m.	2	"	"	"	"	"	+			
		3 p.m.	5	"	"	"	"	"	+			
		5 p.m.	7	"	"	"	"	"	+			
"	15,	7 a.m.	21	"	"	"	"	"	0			
		9 a.m.	23	"	"	"	"	"	0			
		11 a.m.	25	"	"	"	"	"	0			
"	16,		2 days	"	"	"	"	"	0			
"	19,		5	"	"	"	"	"	0			
"	20,		6	"	"	"	"	"	0			
"	21,		7	"	"	"	"	"	0			
"	22,		8	"	"	"	"	"	0			
"	27,		13	"	"	"	"	"	0			
Aug.	3,		20	"	"	"	"	"	0	C. B.	0	
"	9,		26	"	"	"	"	"	0			
"	17,		34	"	"	"	"	"	0	"	"	0
"	24,		41	"	"	"	"	"	0	"	"	0
"	31,		48	"	"	"	"	"	0			
Sept.	2,		50	"	"	"				"	"	0
"	7,		55	"	"	"	"	"	0			
"	14,		62	"	"	"	"	"	0	"	"	0
"	22,		70	"	"	"	"	"	0	"	"	0
"	29,		77	"	"	"	"	"	0	"	"	0
Oct.	5,		83	"	"	"	"	"	0			
"	6,		84	"	"	"				"	"	0
"	13,		91	"	"	"	"	"	0			
"	15,		93	"	"	"				"	"	0
"	21,		99	"	"	"				"	"	0
"	23,		Tryparsamide 0.5 gm. (Provocative dose).									
"	26,						G. P.	0	C. B.	0		
"	30,		Tryparsamide 0.5 gm. (2nd Provocative dose).									
Nov.	2,						G. P.	0	C. B.	0.		

Mondi #75.

Admitted for ambulatory treatment October 22, 1920. 45 kilos.
Cervical lymph glands enlarged. Gland puncture +.
Centrifuged blood +.
Lumbar puncture not done.

1920							
Oct. 22,	11.45 a.m.	Tryparsamide 2.0 gm. (44 mg. per kg.) adm. i.v.					
	3.15 p.m.	3½ hours after treatment					C. B. 0
	4 p.m.	4¼	"	"	"	G. P. 0	
	5 p.m.	5¼	"	"	"		C. B. 0
" 23,	7 a.m.	18¾	"	"	"		" " 0
	9 a.m.	20¾	"	"	"	" " 0	

Victor Makau #77.

Admitted as ambulatory patient October 27, 1920. 57 kilos.
Cervical lymph glands enlarged. Gland puncture +++.
Centrifuged blood +++.
Lumbar puncture 3.8 lymphocytes per cmm. (1 large mononuclear seen.)

1920							
Oct. 28,	9 a.m.	Tryparsamide 1.0 gm. (18 mg. per kg.) adm. i.v.					
	9.15 a.m.	15 minutes after treatment					C. B. +++
	10 a.m.	1 hour	"	"			" " +++
	11 a.m.	2 hours	"	"			" " +
	12 n.	3	"	"	"		" " +
	3.30 p.m.	6½	"	"	"		" " +++
	4 p.m.	7	"	"	"	G. P. +++	
	5 p.m.	8	"	"	"		" " ++
" 29,*	8 a.m.	23	"	"	"	" " 0	
	3 p.m.	30	"	"	"		" " 0

* Patient was quite ill the second day and only a gland puncture could be done at 8 a. m.

Itoma #78.

Admitted for ambulatory treatment October 29, 1920. 52 kilos.
Cervical lymph glands enlarged. Gland puncture ++.
Lumbar puncture deferred. Very advanced stage.

1920												
Oct. 29,	5.30 p.m.	Tryparsamide 1.0 gm. (19 mg. per kg.) adm. i.v.										
" 30,	6.30 a.m.	13	hours	after	treatment					C. B.	0	
	7 a.m.	13½	"	"	"	G. P.	0					
	9 a.m.	15½	"	"	"	"	"	0	"	"	0	

Tuamene #68.

Admitted to lazaret October 5, 1920. 62.5 kilos.
Cervical lymph glands enlarged.
Gland puncture ++. Centrifuged blood ++.
Lumbar puncture 5 lymphocytes per cmm. (1 large mononuclear seen.)

1920												
Oct. 5,	11 a.m.	Tryparsamide 5.0 gm. (80 mg. per kg.) adm. i.v.										
	5 p.m.	6	hours	after	treatment	G. P.	0			C. B.	0	
" 6,	8 a.m.	21	"	"	"	"	"	0				
" 14,		9	days	"	"	"	"	0				
" 21,		16	"	"	"	"	"	0				
" 22,		17	"	"	"					"	"	0
" 28,		23	"	"	"	"	"	0		"	"	0
" 30,		25	"	"	"	"	"	+		"	"	+
Nov. 1,	8 a.m.	Tryparsamide 5.0 gm. (82 mg. per kilo) adm. i.v.										
	11 a.m.	3	hours	after	treatment	G. P.	+					
	12 n.	4	"	"	"					C. B.	+	
	3 p.m.	7	"	"	"					"	"	+
	4 p.m.	8	"	"	"	"	"	0				
	5 p.m.	9	"	"	"					"	"	0
" 2,	8 a.m.	24	"	"	"					"	"	0

The deduction from this series of observations on the effect of a single dose of tryparsamide administered intravenously to a mixed group of patients infected with *Tr. gambiense* is, briefly expressed, as follows: Doses of tryparsamide of 1.0 to 5.0 gm. bring about peripheral sterilization as indicated by negative findings for trypanosomes in lymph gland juice and centrifuged blood in an average of 6 to 12 hours. It would appear that a similar speed of action follows the intramuscular injection of the drug.

Duration of Peripheral Sterilization after Single Doses.

21 patients were treated with single doses of tryparsamide of 0.5 to 5.0 gm. or from 9 to 83 mg. per kilo of body weight and followed with frequent microscopic examinations of lymph gland juice and centrifuged blood to ascertain the permanence of therapeutic effect as expressed by the duration of the peripheral sterilization. Table III summarizes the protocols of the 12 patients who relapsed after single doses of from 0.5 to 5.0 gm. of tryparsamide or from 17 to 83 mg. per kilo of body weight, while Table IV summarizes the records of 9 patients who showed no demonstrable relapse following treatment with single doses of 0.5 to 3.0 gm. or 9 to 68 mg. per kilo. All patients were either in the first period of the disease or at the beginning of the second as shown by the slight excess in the cell count of the spinal fluid, 3 lymphocytes per cmm. being considered normal (10). The drug was administered intravenously except in 4 cases in which the intramuscular route was employed.

TABLE III.

Relapses after Single Doses of Tryparsamide.
Intravenous Administration.

Case No.	Name.	Weight.	Spinal fluid.	Dose.	Dose.	Relapse.	Time after treatment.
		kg.		*gm.*	*mg. per kg.*		*days*
50	Moangolo.....	29.5	Normal.	0.5	17	C. B. + G. P. −	29
48	M'Bondo.....	50	5	1.0	20	C. B. + G. P. +	23
28	Mago.....	33	5	1.0	33	C. B. + G. P. +	19
47	Mondeke.....	61	7.5	2.0	33	C. B. + G. P. +	26
43	Mambula.....	27	7.5	1.0*	37	C. B. + G. P. −	58
36	Sudila.....	46	20	2.0	44	C. B. + G. P. −	50
63	N'Kaka.....	42.5	Normal.	2.0	47	C. B. + G. P. +	17
39	Mulamba.....	55.5	4	3.0*	54	C. B. + G. P. −	45
41	Bajaka.....	53	15	3.0*	57	C. B. + G. P. −	37
42	N'Keta.....	36	10	2.0*	57	C. B. + G. P. +	51
68	Tuamene.....	62.5	5	5.0	80	C. B. + G. P. +	25
53	Ganzema.....	36	3.4 (1 l. m.)	3.0	83	C. B. + G. P. +	21

l. m.—Large mononuclear.
* Intramuscular administration.

In the first group as shown in Table III, there were 2 relapses at 17 and 19 days (Mago #28 and N'Kaka #63), 5 from 21 to 29 days (Ganzema #53, M'Bondo #48, Mondeke #47, Tuamene #68, and Moangolo #50), 2 at 37 and 45 days (Bajaka #41 and Mulamba #39), and 3 from 50 to 58 days (Sudila #36, N'Keta #42, and Mambula #43). The following protocols illustrate the method of determining the time of relapse after treatment.

Moangolo № 50.

Admitted to hospital September 7, 1920. 29.5 kilos.
Cervical lymph glands enlarged. Gland puncture +.
Lumbar puncture normal.

1920												
Sept.	7,	11.30 a.m.	Tryparsamide 0.5 gm. (17 mg. per kg.) adm. i.v.									
"	10,	3 days	after	treatment		G. P.	0					
"	15,	8 "	"	"	"	"	0					
"	17,	10 "	"	"	"	"	0					
"	20,	13 "	"	"	"	"	0					
"	24,	17 "	"	"	"	"	0					
"	25,	18 "	"	"	"	"	0	C. B.	0			
Oct.	1,	24 "	"	"	"	"	0					
"	4,	27 "	"	"	"	"	0	"	"	0		
"	5,	28 "	"	"	"	"	0					
"	6,	29 "	"	"	"	"	+	"	"	+		

M'Bondo № 48.

Admitted to lazaret August 30, 1920. 50 kilos.
Cervical lymph glands enlarged. Gland puncture +++.
Lumbar puncture 5 lymphocytes per cmm.

1920												
Aug.	31,	11.45 a.m.	Tryparsamide 1.0 gm. (20 mg. per kg.) adm. i.v.									
		3.15 p.m.	3½	hours	after	treatment	G. P.	++				
		4.45 p.m.	5	"	"	"	"	"	++			
Sept.	1,	7 a.m.	19	"	"	"				C. B.	0	
		9 a.m.	21	"	"	"	"	"	0			
"	6,		6	days	"	"	"	"	0			
"	11,		11	"	"	"				"	"	0
"	17,		17	"	"	"	"	"	0	"	"	0
"	20,		20	"	"	"	"	"	0			
"	23,		23	"	"	"	"	"	+	"	"	+

Mondeke #47.

Admitted to lazaret August 28, 1920. 61 kilos.
Cervical lymph glands enlarged. G. P. +.
Lumbar puncture 7.5 lymphocytes per cmm.

1920											
Aug. 28, 11	a.m.	Tryparsamide 2.0 gm. (33 mg. per kg.) adm. i.v.									
	4.30 p.m.	5½	hours after treatment	G. P.	+ (1 tryp.)						
	5 p.m.	6	"	"	"	"	"	0	C. B.	0	
Sept. 6,		9	days	"	"	"	"	0			
" 10,		13	"	"	"	"	"	0			
" 11,		14	"	"	"				"	"	0
" 17,		20	"	"	"	"	"	0	"	"	0
" 20,		23	"	"	"	"	"	0			
" 23,		26	"	"	"	"	"	+ (1 tryp.)	"	"	+ (2 tryp.)

Sudila #36.

Admitted for ambulatory treatment August 19, 1920. 46 kilos.
Gland puncture +.
Lumbar puncture 20 lymphocytes per cmm.

1920											
Aug. 19, 5	p.m.	Tryparsamide 2.0 gm. (44 mg. per kg.) adm. i.v.									
" 20, 6	a.m.	13	hours after treatment	G. P.	0						
	6.30 a.m.	13½	"	"	"				C. B.	0	
" 26		7	days	"	"	"	"	0			
" 31,		12	"	"	"	"	"	0			
Sept. 2,		14	"	"	"				"	"	0
" 7,		19	"	"	"	"	"	0			
" 14,		26	"	"	"	"	"	0			
" 21,		33	"	"	"	"	"	0			
" 22,		34	"	"	"				"	"	0
" 28,		40	"	"	"	"	"	0			
Oct. 5,		47	"	"	"	"	"	0			
" 8,		50	"	"	"				"	"	+
" 9,		51	"	"	"	"	"	0			

It so happens that 8 of the 12 relapsed cases, including Tuamene #68 and Ganzema #53, who were given the largest amounts of drug per kilo, came from so called "virulent" districts or villages[1] and that

[1] Certain sections of the Belgian Congo are known as "severe" or "virulent" districts because the infection acquired there is more difficult to influence therapeutically than that of other places.

M'Bondo #48, Mago #28, and Tuamene #68 showed an unusually heavy gland infection at the time of treatment, in fact the most marked of any of the 21 patients considered in these two groups. The spinal fluids of the relapsed cases, moreover, showed greater abnormality than those of the non-relapses (Table IV). Doubtless these factors contribute towards the shortening of the duration of action of the drug but it is not possible to define their effects with accuracy and the point is merely alluded to here.

The duration of negative gland and blood examinations resulting from the intramuscular injection of tryparsamide in 4 patients was appreciably longer than that observed after the intravenous route and since this mode of administration possesses obvious advantages, particularly in field work, abstracts of these cases are given:

Mambula № 43.—Young boy from a "virulent" region. Severe lymph gland infection. Spinal fluid, 7.5 lymphocytes per cmm. Treated with 1.0 gm. tryparsamide (37 mg. per kilo of body weight) in 3.0 cc. of water, injected into the deep muscles of the left buttock. Slight transitory local tenderness lasting a few hours.

Gland puncture negative 21½ hours after treatment; 12 subsequent microscopic gland examinations and 5 examinations of centrifuged blood were negative during 56 days. A second lumbar puncture 34 days after treatment showed a normal cell count (2.1 lymphocytes per cmm.)—a decrease from the initial count.

A relapse was finally detected 58 days after treatment by the finding of 1 trypanosome in the centrifuged blood. Gland puncture was negative.

Mulamba № 39.—Soldier who had been in a "virulent" region during the previous year. Moderate lymph gland infection; spinal fluid contained 4 lymphocytes per cmm. Treated with 3.0 gm. tryparsamide (54 mg. per kilo) in 10 cc. of water injected deeply into muscles of the right buttock. The following morning, 14½ hours after treatment, gland puncture was negative and there was no induration or tenderness of the injection area. Patient reported slight local discomfort after the injection lasting a few hours. No cells were found in the spinal fluid examined 35 days after treatment, contrasting with the 4 per cmm. before treatment. 9 gland punctures and 3 examinations of centrifuged blood during the succeeding 42 days were negative but on the 45th day after treatment, one trypanosome was found in the centrifuged blood. Gland puncture negative.

Bajaka № 41.—Adult man from a "virulent" region. Severe lymph gland infection; spinal fluid contained 15 lymphocytes per cmm. Treated with 3.0 gm. tryparsamide in 9 cc. of water (57 mg. per kilo), injected deeply into the muscles of the left buttock. Gland puncture negative 6 hours later at which time patient complained of soreness in the injection area. During the next 2 days,

soreness disappeared and 6 days after injection, there was only a slight induration and slight tenderness on deep pressure of this area.

During 35 days after treatment, the microscopic examination of 8 gland punctures and 3 centrifuged bloods were negative but on the 37th day, 2 trypanosomes were found in the centrifuged blood. Gland puncture was negative.

A second examination of the spinal fluid 35 days after treatment showed 7.5 lymphocytes—a 50 per cent decrease—but 2 large mononuclear cells were also seen indicating the continued involvement of the cerebrospinal system.

N'Keta # 42.—Young woman from a "virulent" region suffering from extensive craw-craw. Severe lymph gland infection. Spinal fluid contained 10 lymphocytes per cmm. Treated with 2.0 gm. tryparsamide (57 mg. per kilo) in 6.0 cc. of water injected deeply into the muscles of the left buttock. Gland puncture negative 7 hours later.

During the following week, injection area became considerably indurated and quite tender although patient was able to walk; there was considerable loss of weight (2.5 kilos), a rapid pulse, subnormal temperature and patient appeared ill. By the end of the second week after treatment, there was but slight residual tenderness on deep pressure, very little induration of a small area and patient's general condition had improved.

Microscopic examination of 9 specimens of lymph gland juice and 5 of centrifuged blood over a period of 48 days were negative but 51 days after treatment 1 trypanosome was found in both the lymph gland and centrifuged blood examinations.

A second spinal fluid examination 34 days after treatment showed a normal cell content (0.6 lymphocytes per cmm.), contrasting with the original count of 10 lymphocytes per cmm.

Several points of interest are brought out in the above four abstracts: In only 1 patient was there any considerable discomfort lasting more than a few hours in the area of intramuscular injection. This patient had a marked case of craw-craw and in addition was indescribably dirty. Although the skin of the injection area was cleansed with iodine and alcohol, probably an infection of some kind was set up in the deep muscles, resulting in a tender area of induration which was absorbed within 2 weeks. Similar effects following the intramuscular injection of other drugs are not unusual under such circumstances according to the experience of the physicians in Léopoldville. Next. the time of initial therapeutic action of the drug is comparable with that observed after intravenous administration of the drug. Thus in these 4 cases with severe lymph gland infection, negative punctures were obtained within $21\frac{1}{2}$ hours and, in the cases in which earlier

examination could be made, 6 to 14½ hours after intramuscular injections of doses of 54 and 57 mg. per kilo of body weight. Moreover, the action of single doses of from 1.0 to 3.0 gm. (37 to 57 mg. per kilo of body weight) lasted from 37 to 58 days. These 4 patients came from a region in which the trypanosomal disease is recognized as more difficult to influence than in other places and, although all 4 patients ultimately suffered relapses, it is noteworthy that moderate sized doses controlled the recurrence of lymph gland and blood infection for from 5 to 8 weeks. Finally, a distinct effect was observed in the spinal fluid in that the cell count was decreased in all four patients, the second lumbar puncture being done 5 weeks after treatment. Thus, Mambula #43, showed a decrease from 7.5 to 2.1 lymphocytes per cmm.; Mulamba #39 from 4.0 to 0 lymphocytes; N'Keta #42 from 10 to 0.6 lymphocytes and Bajaka #41 from 15 to 7.5 lymphocytes per cmm. Although the patients treated intramuscularly are comparatively few in number, the results obtained are clear cut and suggest that with this method of administration the drug may remain available in a biologically active state for a period of time perhaps exceeding that of the intravenous route.

TABLE IV.

Non-Relapses after Single Doses of Tryparsamide. Intravenous Administration.

Case No.	Name.	Weight.	Spinal fluid.	Dose.	Dose.	Non-relapse.	Time after treatment.
		kg.		*gm.*	*mg. per kg.*		*days*
40	Esombo.....	51	Normal.	0.5	9	C. B. — G. P. —	109
38	Daie.....	46	(?)R.B.C.	1.0	22	C. B. — G. P. —	40*
30	Mafuala.....	39.6	5	1.0	25	C. B. — G. P. —	75
46	Bokao.....	50	5	2.0	40	C. B. — G. P. —	67
5	Mondongo.....	47	7.5	2.0	43	C. B. — G. P. —	111
45	K. Georges.....	38.5	Normal.	2.0	52	C. B. — G. P. —	68
37	Bukasa.....	54.5	12.5	3.0	55	C. B. — G. P. —	69
51	Gwamuntele....	44	Normal.	3.0	67	C. B. — G. P. —	54
31	Lubaki.....	44.5	Normal.	3.0	68	C. B. — G. P. —	75

* Considered a relapse because of probable increase of cell content of spinal fluid, 37 days after treatment.

Table IV summarizes the records of 9 patients treated intravenously with single doses of tryparsamide of from 0.5 to 3.0 gm. or from 9 to 68 mg. per kilo of body weight, in whom return of trypanosomes in the lymph glands or blood from 40 to 111 days after treatment was not demonstrable. As will be seen by comparing Tables III and IV, similar sized doses were given to the two groups of patients. The type and stage of the infection of the non-relapsed patients were as follows: 4 showed slightly abnormal spinal fluids, Mafuala #30, 5; Bokao #46, 5; Mondongo #5, 7.5; and Bukasa #37, 12.5 lymphocytes per cmm.; while 4 had normal spinal fluid cell counts—Esombo #40, K. Georges #45, Gwamuntele #51, and Lubaki #31. In one instance, Daie #38, the spinal fluid was contaminated with blood so that an accurate cell count could not be made. Although trypanosomes were readily found by microscopic examination of the lymph gland juice or of centrifuged blood before treatment, in general, the lymph gland infection was not a heavy one except in the case of Bukasa #37. In addition, none of these patients had come, as far as could be ascertained, from the "virulent" regions referred to. Hence, these cases may represent a less advanced period of the disease and a somewhat less virulent type of infecting organism than the majority of patients listed in Table III.

Although patient Daie #38 is included in Table IV because no trypanosomes were found in the lymph gland juice, centrifuged blood, or centrifuged spinal fluid for 40 days after treatment with 1.0 gm. of tryparsamide, the probable increase in the cells of the spinal fluid, together with an increased pulse rate, loss of weight, and negative malarial examination made it seem probable that a relapse had really occurred and consequently a system of repeated dose therapy was begun.

With this exception, the 8 patients who were under close observation for 54 to 111 days and who received a single dose of tryparsamide of from 9.0 to 68 mg. per kilo suffered no relapse as far as could be discovered. For comparison, one other case may be mentioned in which no relapse occurred although the period of observation was only 26 days. This patient, Talamene #73, came from an active epidemic area with a severe lymph gland infection; his spinal fluid was normal. He was given 4.0 gm. of tryparsamide or 109 mg. per

kilo intravenously. Gland puncture was negative 14 hours and centrifuged blood 15 hours after treatment. Subsequent examinations were also negative for 26 days but as it was feared that he would run away, the drug was repeated systematically. The observations on this patient may be compared with those on 2 others, Tuamene #68 and Ganzema #53, who relapsed (Table III); the lymph gland infection of Talamene #73 and Tuamene #68 were both severe, while Ganzema #53 came from a village known to furnish particularly difficult cases. They were given doses of 80 and 83 mg. per kilo which produced peripheral sterilization for 25 and 21 days respectively. In the case of Talamene #73, the larger dose of 109 mg. per kilo resulted in negative lymph gland and blood examinations for at least 26 days.

The following protocols illustrate the time and frequency of examinations carried out with the patients who showed no return of trypanosomes during the period of observation:

Esombo # 40.

Admitted for ambulatory treatment August 24, 1920. 51.0 kilos.
Cervical lymph glands enlarged. Gland puncture +.
Lumbar puncture normal.

1920										
Aug. 24, 4.30 p.m.	Tryparsamide 0.5 gm. (9.0 mg. per kg.) adm. i.v.									
" 25, 7.30 a.m.	15 hours after treatment				G.	P.	0			
Sept. 1,	8 days	"	"	"	"	"	0			
" 7,	14	"	"	"	"	"	0			
" 13,	20	"	"	"				C.	B.	0
" 14,	21	"	"	"	"	"	0			
" 21,	28	"	"	"	"	"	0			
" 22,	29	"	"	"				"	"	0
" 25,	32	"	"	"	"	"	0			
" 28,	35	"	"	"	"	"	0			
Oct. 5,	42	"	"	"	"	"	0			
" 8,	45	"	"	"				"	"	0
" 12,	49	"	"	"	"	"	0			
" 19,	56	"	"	"	"	"	0	"	"	0
" 20,	57	"	"	"				"	"	0
" 21,	58	"	"	"	"	"	0			
" 26,	63	"	"	"				"	"	0
Dec. 11,	109	"	"	"				"	"	0

Mafuala № 30.

Admitted to lazaret August 16, 1920. 39.6 kilos.
Cervical lymph glands enlarged. Gland puncture +.
Centrifuged blood +. Lumbar puncture normal.

1920

Aug. 16, 11 a.m. Tryparsamide 1.0 gm. (25 mg. per kg.) adm. i.v.
5 p.m. 6 hours after treatment G. P. 0 C. B. 0
" 17, 8 a.m. 21 " " " " " 0
" 19, 3 days " " " " " 0
" 24, 8 " " " " " 0
" 31, 15 " " " " " 0
Sept. 3, 18 " " " " " 0
" 6, 21 " " " " " 0
" 14, 29 " " " " " 0
" 20, 35 " " " " " 0
" 23, 38 " " " " " 0 " " 0
" 27, 42 " " " " " 0
Oct. 1, 46 " " " " " 0
" 6, 51 " " " " " 0
" 12, 57 " " " " " 0
" 15, 60 " " " " " 0
" 19, 64 " " " " " 0
" 20, 65 " " " " " 0
" 21, 66 " " " " " 0
" 25, 70 " " " " " 0
" 30, 75 " " " " " 0 " " 0

Bukasa № 37.

Admitted to lazaret August 21, 1920. 54.5 kilos.
Cervical lymph glands enlarged. Gland puncture ++.
Lumbar puncture 12.5 lymphocytes per cmm.

1920

Aug. 21, 5.30 p.m. Tryparsamide 3.0 gm. (55 mg. per kg.) adm. i.v.
" 22, 6 a.m. 12½ hours after treatment G. P. 0
7 a.m. 13½ " " " C. B. 0
Sept. 3, 13 days " " " " 0
" 10, 20 " " " " " 0
" 17, 27 " " " " " 0
" 20, 30 " " " " " 0
" 28, 38 " " " " " 0
Oct. 4, 44 " " " " " 0
" 6, 46 " " " " " 0
" 7, 47 " " " " " 0
" 14, 54 " " " " " 0
" 15, 55 " " " " " 0
" 19, 59 " " " " " 0
" 25, 65 " " " " " 0
" 29, 69 " " " " " 0

Lubaki № 31.

Admitted to lazaret August 16, 1920. 44.5 kilos.
Cervical lymph glands palpable. Gland puncture 0.
Centrifuged blood +. Lumbar puncture normal.

1920												
Aug.	16,	11.15 a.m.	Tryparsamide 3.0 gm. (68 mg. per kg.) adm. i.v.									
"	17,	8.30 a.m.	21 hours after treatment							C. B.	0	
"	24,		8 days	"	"		G. P.	0	"	"	0	
Sept.	4,		19	"	"	"			"	"	0	
"	14,		29	"	"	"	"	"	0	"	"	0
"	23,		38	"	"	"	"	"	0	"	"	0
"	30,		45	"	"	"	"	"	0			
Oct.	7,		52	"	"	"				"	"	0
"	12,		57	"	"	"	"	"	0			
"	15,		60	"	"	"				"	"	0
"	21,		66	"	"	"	"	"	0			
"	25,		70	"	"	"				"	"	0
"	30,		75	"	"	"	"	"	0	"	"	0

TABLE V.

Summary of Relapses and Non-Relapses after Single Doses of Tryparsamide.

Doses of from 9 to 33 mg. per kg. of body weight.			Doses of from 37 to 52 mg. per kg. of body weight.			Doses of from 54 to 83 mg. per kg. of body weight.		
Name.	Dose.	Result.	Name.	Dose.	Result.	Name.	Dose.	Result.
	gm.	*days*		*gm.*	*days*		*gm.*	*days*
40 Esombo....	0.5	0 109	43 Mambula...	1.0	+ 58	39 Mulamba...	3.0	+ 45
50 Moangolo..	0.5	+ 29	46 Bokao.....	2.0	0 67	37 Bukasa.....	3.0	0 69
48 M'Bondo...	1.0	+ 23	5 Mondongo..	2.0	0 111	41 Bajaka.....	3.0	+ 37
30 Mafuala...	1.0	0 75	36 Sudila.....	2.0	+ 50	42 N'Keta.....	2.0	+ 51
28 Mago.....	1.0	+ 19	63 N'Kaka	2.0	+ 17	51 Gwamuntele	3.0	0 54
47 Mondeke...	2.0	+ 26	45 K. Georges.	2.0	0 68	31 Lubaki.....	3.0	0 75
						68 Tuamene...	5.0	+ 25
						53 Ganzema...	3.0	+ 21
2 Non-relapses.			3 Non-relapses.			3 Non-relapses.		
4 Relapses in average of 24 days or 3 weeks.			3 Relapses in average of 42 days or 6 weeks.			5 Relapses in average of 36 days or 5 weeks.		

In considering the length of time of peripheral sterilization in relation to the size of the dose, it will be of assistance to glance at Table V which groups together the instances of relapse and non-

relapse of Tables III and IV according to the amount of drug administered. Daie #38 is not included since we were uncertain of the result. The first column of Table V contains the results of the treatment of 6 patients with doses of from 9 to 33 mg. per kilo of body weight or, with the usual size of the native in the lower Congo, a dose of from 0.5 to 2.0 gm. Of these 6 patients, 4 suffered a relapse in from 19 to 29 days or on an average of 24 days, a little over a 3 weeks' period. The 2 non-relapsed patients, of whom one had received the small dose of 9.0 mg. per kilo of body weight, were still clear 109 and 75 days after treatment. In the second group, 6 patients were treated with amounts ranging from 37 to 52 mg., usually a 2.0 gm. dose, and 3 relapsed in from 17 to 58 days or on an average of 42 days or 6 weeks. The instances of non-relapse in this group had been under observation respectively for 67, 68, and 111 days. In the third group comprising 8 patients who received 2.0, 3.0, and 5.0 gm. doses, or from 54 to 83 mg. per kilo of body weight, 5 relapsed in from 21 to 51 days or on an average of 36 days or practically 5 weeks. The 3 non-relapsed patients were clear 54, 69, and 75 days after treatment.

Speaking generally, there is apparently not a great deal of difference between the results obtained with what might be called average doses, that is, 2.0 and 3.0 gm.—as shown in columns 2 and 3 of Table V—since the average negative period for trypanosomes was 6 and 5 weeks respectively. There is a distinct shortening of this period, however, after the smaller doses of 0.5 and 1.0 gm. as shown by the average figure of 3 weeks. Thus it appears that once a certain amount of drug is biologically available for therapeutic action, it may be unnecessary to increase the size of the dose above this amount which represents a favorable ratio between rate of absorption and elimination and duration of action on the parasites.

However, from the point of view of planning a system of repeated doses, especially with a new or little tried drug, one is not justified in the beginning in working with averages of duration of peripheral sterilization. The shortest period of sterilization was 17 days after a dose of 2.0 gm. or 47 mg. per kilo of body weight and accordingly two principal systems of therapy were planned. These consisted in giving average doses of 2.0, 3.0, and 4.0 gm. according to the weight

of the patient at weekly or fortnightly intervals for 4 to 8 injections. A number of patients were thus treated toward the end of our stay in the Congo, the results of which will be sent to us by the Belgian physician in charge of the cases in Léopoldville.

Effect of Single Doses upon the Spinal Fluid.

The effect of a drug upon the spinal fluid of patients suffering from trypanosomiasis is of great importance, since the natural evolution of the disease is toward involvement of the cerebrospinal system. The ultimate outcome of certain cases is at once problematical if the therapeutic field of action of a drug is limited, practically speaking, to that of so called peripheral sterilization, while if the cerebrospinal system is included in its sphere of action and the effect of its administration is reflected in the spinal fluid, the therapeutic possibilities of the drug are at once extended. Thus, in cases of human trypanosomiasis in the first stage in which the spinal fluid is normal, it is essential to ascertain if that condition continues so or whether, despite the negative blood and lymph gland examination, the cerebrospinal axis may be invaded.

Moreover, in those cases in which some invasion has already taken place as indicated by a slight pleocytosis, the selection of a drug which tends to limit the further progress of the disease is urgently called for. Finally, in the advanced cases with more or less cerebrospinal involvement and alteration of the spinal fluid, such an agent is obviously indicated.

Broden and Rodhain (8, 9, and 10) have shown that in human trypanosomiasis, the cell content of the spinal fluid may be looked upon as an index of the extent of cerebrospinal involvement, thus serving as a diagnostic criterion and a therapeutic guide. They consider that a normal spinal fluid contains no more than 3 lymphocytes (small mononuclears) per cmm.;[2] and that in the cerebrospinal stage of the disease, the first abnormality to be noted is an increase in the lymphocytes which is followed in the more advanced cases by

[2] The cells are counted directly by means of a Fuchs-Rosenthal chamber, immediately after the withdrawal of a few cubic centimeters of spinal fluid which is not centrifuged.

the appearance of medium sized mononuclear cells and so called mulberry cells or large vacuolated mononuclear cells (*cellules en mûre*). The very advanced cases, on the other hand, may show extremely large vacuolated cells, rare polymorphonuclear cells with eosinophilic granules and cells which apparently have become detached from the surface of the meninges. Although minor variations in the cell count of the spinal fluid occurs from time to time, in general, once the cerebrospinal system has become affected, progress tends to be continuous with increasing abnormality of the spinal fluid. Trypanosomes may be found in the centrifuged spinal fluid of the more advanced cases, but it is not always practical to withdraw the necessary amounts of fluid for this procedure. The nature of the infection in these cases is usually readily established by gland puncture or examination of centrifuged blood so that demonstration of trypanosomes in the spinal fluid was not attempted as a routine measure.

The effect, therefore, of the administration of tryparsamide upon the spinal fluid was carefully followed. The usual procedure was: A lumbar puncture was done before treatment and from 3 to 10 cc. of fluid removed and the cells counted. The period of the disease was based on the results obtained irrespective of other clinical findings. The manner of treatment was also determined by this procedure since when a moderate or marked alteration in the cell content was found more vigorous treatment than a single dose of the drug was indicated. A second lumbar puncture was done about 5 weeks later, and when possible, again at some more remote date; more frequent punctures were not made as the patients were ambulatory, and, in addition, we wished to avoid so called drainage changes which might result from such a procedure.

In Table VI are given the cell counts of 14 patients before and after treatment with single doses of tryparsamide. This number includes 9 patients in whom no return of trypanosomes in the blood or lymph glands was detected during the period of observation after treatment and 5 who eventually relapsed but whose second lumbar puncture was done before this occurred. The cell content of the spinal fluids was normal or showed a slight increase up to 20 lymphocytes per cmm. Single does of 0.5 to 3.0 gm. or 9 to 68 mg. per kilo of body weight were administered intravenously to 10 and intramuscularly to 4 patients.

The second lumbar puncture was done approximately 5 weeks after treatment in all the patients except Sudila #36 and Gwamuntele #51, in whom the interval between the first and second lumbar puncture was 40 and 47 days and in the case of Mondongo #5, in which it was 26 days. It will be noted that with the exception of Daie #38, the second examinations showed no increase in the cell

TABLE VI.

Cell Content of Spinal Fluid after Single Doses.

Case No.	Name.	Weight.	Dose.	Dose.	Cell content before treatment.	Cell content after treatment.	Interval between treatment and 2nd L. P.	Remarks.	
		kg.	*gm.*	*mg. per kg.*			*days*		*days*
40	Esombo.....	51	0.5	9	Normal.	Normal.	35	No relapse	109
38	Daie.....	46	1.0	22	(?)R.B.C.	35 (1 l.m.)	37	No relapse (?)†	
30	Mafuala....	39.6	1.0	25	5	4.6	37	No relapse	75
43	Mambula...	27	1.0*	37	7.5	Normal.	34	Relapse	58
46	Bokao.....	50	2.0	40	5	Normal.	34	No relapse	67
5	Mondongo..	47	2.0	43	7.5	5	26	No relapse	111
						3 (2 l.m.)	93		
36	Sudila.....	46	2.0	44	20	Normal.	40	Relapse	50
45	K. Georges..	38.5	2.0	52	Normal.	Normal.	34	No relapse	68
39	Mulamba...	55.5	3.0*	54	4	Normal.	35	Relapse	45
37	Bukasa.....	54.5	3.0	55	12.5	Normal.	38	No relapse	69
42	N'Keta.....	36	2.0*	57	10	Normal.	34	Relapse	51
41	Bajaka	53	3.0*	57	15	7.5 (2 l.m.)	35	Relapse	37
51	Gwamuntele.	44	3.0	67	Normal.	Normal.	47	No relapse	54
31	Lubaki	44.5	3.0	68	Normal.	Normal.	37	No relapse	75

l.m.—Large mononuclears.

* Intramuscular administration.

† Cf. Table IV.

content; but 4, Esombo #40, K. Georges #45, Gwamuntele #51, and Lubaki #31, continued to be normal; and 2, Mafuala #30 and Mondongo #5, whose original counts were 5 and 7.5 lymphocytes per cmm., showed practically no change, 4.6 and 5 lymphocytes per cmm. respectively. On the other hand, 6 patients whose spinal fluids showed slight changes before treatment (Mambula #43, 7.5; Bokao

#46, 5; Sudila #36, 20; Mulamba #39, 4; Bukasa #37, 12.5; N'Keta #42, 10) all had normal cell counts at the time of the second examination. One patient, Bajaka #41, with an initial cell content of 15, showed 7.5 lymphocytes 35 days after treatment but as 2 large mononuclears were seen in the counting chamber, it is probable that the influence of the drug was less marked than in the other cases. This patient came from a "virulent" region and at the time of treatment had a very severe lymph gland infection. A third lumbar puncture done 93 days after a single dose of 2.0 gm. in one patient, Mondongo #5, showed 3 lymphocytes or a normal count but, as in the case of Bajaka #41, 2 large mononuclears having been seen, according to the standards established by Broden and Rodhain, the possibility of some degree of cerebrospinal involvement must be considered. Finally, one patient, Daie #38, showed what was apparently a progression of the disease. An accurate initial cell count was impossible because the spinal fluid was contaminated with blood but the second cell count, done 37 days after treatment with the small dose of 22 mg. per kilo of body weight, showed 35 lymphocytes per cmm., and 1 large mononuclear in the counting chamber. No trypanosomes were found in centrifuged specimens of the spinal fluid and repeated examinations of lymph glands and blood had been negative since treatment. As there had been a loss of 1.5 kilos in weight and no malarial parasites were found and as 4 days before the second lumbar puncture the pulse had risen from the usual rate of 72 to 80 to 92, this man was transferred to the group of patients being treated with repeated doses of tryparsamide.

The observations just described indicate that in the first period of the disease or in the very early stages of the second period, single small and medium doses of tryparsamide will in nearly all cases be followed by beneficial effects as indicated by the influence upon the cell content of the cerebrospinal fluid. Perhaps it may be permissible to go even further than this and to state that in these early stages an arrest of the disease has been achieved although it must be remembered that there is no criterion of the time of occurrence of cerebrospinal involvement and no prediction of the duration of the observed effect can be made.

Effect of Single Doses upon the Blood.

The blood condition of natives suffering from trypanosomiasis is often complicated by the presence of one or more other infections. In the lower and middle Congo regions, almost every native shows an enlarged spleen and chronic malaria; moreover, skin infections, filariasis, intestinal parasites, and amœbiosis are common, and venereal disease is frequent. Keeping in mind these facts, most authorities agree that the blood changes in human trypanosomiasis consist of diminution of red cells, decrease in hemoglobin, and normal or slightly subnormal white cells associated with a relative increase of mononuclear cells and a decrease of granular leukocytes.

Blood counts were followed in 12 patients in the early period of the disease or at the period of slight abnormality of the cerebrospinal fluid. Single doses of tryparsamide, ranging from 0.5 to 5.0 gm. were given intravenously to 10 and intramuscularly to 2 patients (Table VII) including 8 non-relapsed subjects who had negative lymph gland and blood examinations during the period of observation; 1 patient, who had not relapsed 26 days after treatment but who was transferred to the series of repeated doses at this time and 3 relapsed patients from whom a second blood count had been obtained before trypanosomes were again found in the lymph glands or blood. The time of the second blood examination in relation to the first, taken on the day of treatment, ranged from 26 to 63 days.

At the first count, there was in these cases a marked decrease in hemoglobin and a somewhat less decrease in the red cells or an anemia of the secondary type. Only 2 patients showed an original increased or normal red cell count; 4 showed counts of 4 million; 4 showed counts between 3 and 4 million, and 2 patients showed only 2 and $2\frac{1}{2}$ million red cells.

The injection of a single dose of tryparsamide (0.5 to 5.0 gm.) was followed in 41 to 63 days by an increase in the number of red cells in 7 patients ranging from 10 to 60 per cent as shown in Table VIII. One patient, Tuamene #68, showed an increase of 15 per cent in 28 days, while in two others. Bukasa #37 and Mondongo #5, the increase was 47 and 60 per cent in 60 and 41 days respectively. In 3 patients with initial counts of 4 million, Talamene #73, Lubaki #31, K. Georges

TABLE VII.

Changes in Blood Counts after Single Doses of Tryparsamide.

Case No.	Name.	Dose adm. i. v.	1st blood count.	2nd blood count.	Change.	Time after treatment of 2nd count.
					per cent	*days*
40	Esombo.....	0.5 gm.	R. B. C. 5,760,000	6,400,000	+10	63‡
	51 kg.	9.0 mg.	Hb. 55 %	70 %	+27	
		per kg.	W. B. C. 10,800	9,600	−11	
30	Mafuala	1.0 gm.	R. B. C. 4,960,000	4,048,000	−18	37§
	39.6 kg.	25 mg.	Hb. 65 %	70 %	+ 8	
		per kg.	W. B. C. 6,400	8,000	+20	
				4,480,000†	−10†	66†
				65 %	−	
				8,000	+20	
43	Mambula	1.0 gm.*	R. B. C. 4,000,000	4,800,000	+20	48‡
	27 kg.	37 mg.	Hb. 60 %	80 %	+33	
		per kg.	W. B. C. 4,200	6,000	+43	
46	Bokao	2.0 gm.	R. B. C. 3,840,000	4,640,000	+21	54
	50 kg.	40 mg.	Hb. 60 %	70 %	+17	
		per kg.	W. B. C. 5,000	6,600	+32	
5	Mondongo.....	2.0 gm.	R. B. C. 2,000,000	3,200,000	+60	41‖
	47 kg.	43 mg.	Hb. 35%	55 %	+57	
		per kg.	W. B. C. 5,400	9,800	+81	
				3,360,000†	+68†	105†
				55 %	+57	
				12,000	+122	
45	K. Georges.....	2.0 gm.	R. B. C. 4,032,000	4,000,000	−	56‡
	38.5 kg.	52 mg.	Hb. 50 %	60 %	+20	
		per kg.	W. B. C. 8,200	9,000	+10	
37	Bukasa.....	3.0 gm.	R. B. C. 2,490,000	3,656,000	+47	60
	54.5 kg.	55 mg.	Hb. 40 %	70 %	+75	
		per kg.	W. B. C. 5,200	6,000	+15	
42	N'Keta	2.0 gm.*	R. B. C. 3,200,000	3,200,000	−	30**
	36 kg.	57 mg.	Hb. 35 %	45 %	+29	
		per kg.	W. B. C. 4,000	7,600	+90	

TABLE VII—*Concluded.*

Case No.	Name.	Dose. adm. i. v.	1st blood count.	2nd blood count.	Change.	Time after treatment of 2nd count.
					per cent	*days*
51	Gwamuntele....	3.0 gm.	R. B. C. 3,200,000	4,000,000	+25	51‡
	44 kg.	67 mg.	Hb. 40 %	55 %	+38	
		per kg.	W. B. C. 3,600	5,000	+39	
31	Lubaki.....	3.0 gm.	R. B. C. 4,000,000	4,000,000	—	37††
	44.5 kg.	68 mg.	Hb. 65 %	70 %	+8	
		per kg.	W. B. C. 4,700	5,600	+19	
				4,800,000†	+20†	70†
				65 %	—	
				8,000	+70	
68	Tuamene.....	5.0 gm.	R. B. C. 3,680,000	4,240,000	+15	28
	62.5 kg.	80 mg.	Hb. 50 %	60 %	+20	
		per kg.	W. B. C. 8,000	8,000	—	
73	Talamene.....	4.0 gm.	R. B. C. 4,000,000	4,000,000	—	26
	36.5 kg.	109 mg.	Hb. 50 %	65 %	+30	
		per kg.	W. B. C. 4,000	6,000	+50	

* Administered intramuscularly.
† 3rd blood count.
‡ Estivo-autumnal malaria.
§ Estivo-autumnal malaria (?).
‖ Multiple skin ulcers; filariasis; chronic amœbiosis (?).
** Craw-craw; intramuscular abscess.
†† Filariasis.

#45, and in one, N'Keta #42, with a count of 3 million red cells, there was no change noted after 26 to 56 days; 2 of these patients, K. Georges #45 and N'Keta #42, suffered from acute concurrent infections during this period; Lubaki #31 ultimately showed an increase of 20 per cent, 70 days after treatment; and in the case of Talamene #73 the interval between the counts was comparatively short, 26 days. One patient, Mafuala #30, whose first count was 5 million red cells, showed a decrease of 18 per cent in 37 days but in a third count 66 days after treatment the decrease amounted to 10 per cent.

TABLE VIII.

Changes in the Red Blood Cell Counts of Patients Treated with Single Doses of Tryparsamide.

Case No.	Name.	Dose.	Original R. B. C. before treatment.	R. B. C. after treatment.	Change.	Interval between counts.
		mg. per kg.			*per cent*	*days*
5	Mondongo	43	2,000,000	3,200,000 3,360,000	+60 +68	41 105
37	Bukasa	55	2,490,000	3,656,000	+47	60
42	N'Keta	57	3,200,000	3,200,000	—	30
51	Gwamuntele	67	3,200,000	4,000,000	+25	51
68	Tuamene	80	3,680,000	4,240,000	+15	28
46	Bokao	40	3,840,000	4,640,000	+21	54
43	Mambula	37	4,000,000	4,800,000	+20	48
31	Lubaki	68	4,000,000	4,000,000 4,800,000	— +20	37 70
73	Talamene	109	4,000,000	4,000,000	—	26
45	K. Georges	52	4,032,000	4,000,000	—	56
30	Mafuala	25	4,960,000	4,048,000 4,480,000	−18 −10	37 66
40	Esombo	9	5,760,000	6,400,000	+10	63

TABLE IX.

Changes in Hemoglobin Content of Blood after Single Doses of Tryparsamide.

Case No.	Name.	Dose.	Original hemoglobin content before treatment.	Hemoglobin content after treatment.	Change +.	Interval between examinations.
		mg. per kg.	*per cent*	*per cent*	*per cent*	*days*
5	Mondongo	43	35	55	57	41
42	N'Keta	57	35	45	29	30
37	Bukasa	55	40	70	75	60
51	Gwamuntele	67	40	55	38	51
45	K. Georges	52	50	60	20	56
68	Tuamene	80	50	60	20	28
73	Talamene	109	50	65	30	26
40	Esombo	9	55	70	27	63
43	Mambula	37	60	80	33	48
46	Bokao	40	60	70	17	54
30	Mafuala	25	65	65	—	66
31	Lubaki	68	65	65	—	70

The hemoglobin content of the blood, determined by a Sahli hemoglobinometer, was generally increased after treatment with tryparsamide as shown in Table IX. In 10 of the patients in this group, the hemoglobin content rose from 17 to 75 per cent during periods of from 26 to 63 days after single dose treatment while 2 patients each with an original hemoglobin of 65 per cent showed no change. As might be expected, the greatest gains were in those patients with the lowest hemoglobin. Thus, in the first 4 cases with 35 and 40 per cent hemoglobin, the average gain was 50 per cent and in 6

TABLE X.

Changes in the White Blood Cell Counts after Single Doses of Tryparsamide.

Case No.	Name.	Dose.	Original W. B. C. before treatment.	W. B. C. after treatment.	Change +.	Interval between counts.
		mg. per kg.			*per cent*	*days*
51	Gwamuntele.....	67	3,600	5,000	39	51
42	N'Keta.....	57	4,000	7,600	90	30
73	Talamene.....	109	4,000	6,000	50	26
43	Mambula.....	37	4,200	6,000	43	48
31	Lubaki.....	68	4,700	5,600 8,000	19 70	37 70
46	Bokao.....	40	5,000	6,600	32	54
37	Bukasa.....	55	5,200	6,000	15	60
5	Mondongo.....	43	5,400	9,800 12,000	81 122	41 105
30	Mafuala.....	25	6,400	8,000 8,000	20 20	37 66
68	Tuamene.....	80	8,000	8,000	—	28
46	K. Georges.....	52	8,200	9,000	10	56
40	Esombo.....	9	10,800	9,600	−11	63

patients with original values of 50 to 60 per cent the increase was 25 per cent in an average of 46 days.

No direct relation was detected between the size of the dose of tryparsamide and the changes in the red cells or the hemoglobin; it is probable, however, that such improvement as takes place results from the trypanocidal action of the drug.

The initial leucocyte counts of this group were less than 8,000 in 9 of the 12 patients; there were 2 counts of 8,000 and 8,200 and one of 10,800 (Table X). Examinations made at varying intervals of time

after treatment with single doses of tryparsamide showed an increase of white blood cells of from 10 to 122 per cent in 10 patients, no change in an original count of 8,000 cells per cmm. and a 11 per cent decrease in the patient who had an original slight leucocytosis.

The upshot of the observations of these patients is that, despite other concurrent infections, a tendency is to be discerned toward a normal blood picture once the trypanosomal infection is controlled for even a few weeks by a single dose of tryparsamide.

Effect of Single Doses upon the Weight.

The effect upon the weight of all patients treated with tryparsamide was followed and the observations made on 21 patients treated with single doses are shown in Table XI. These patients were in the first period or in the beginning of the second stage of the disease as indicated by the cell content of the spinal fluid and were treated with single doses of from 0.5 to 5.0 gm. given intravenously in 17 and intramuscularly in 4 cases. The group includes 9 non-relapsed cases in whom no trypanosomes were demonstrated microscopically in lymph glands, centrifuged blood or centrifuged spinal fluid, 11 relapsed cases and 1 in whom the result was uncertain.

Considering the group as a whole, 5 patients showed no change of weight in 3 to 12 weeks, 2 showed a loss of 0.5 and 1.5 kilos respectively in 3 and 6 weeks and 14 showed a gain of 0.5 to 8.4 kilos in from 3 to 10 weeks after treatment. Generally speaking, patients who gained little or no weight had intercurrent infections or suffered an ultimate relapse.

Of the 14 patients who gained weight, 7 remained negative during the period of observation and 7 ultimately relapsed. These cases are contrasted in Table XII. The increase in weight of the 7 non-relapsed patients ranged from 0.5 to 8.4 kilos or an average of 3.3 kilos in 4 to 10 weeks. The relapsed patients on the other hand showed smaller gains ranging from 0.5 to 2.5 kilos or an average of 1.4 kilos in 3 to 8 weeks.

General Effects Following Single Doses.

The preceding sections of this paper have dealt with a series of observations carried out on a number of patients suffering with

trypanosomiasis treated with single doses of tryparsamide. These observations consisted of the results of microscopic examinations for trypanosomes in aspirated lymph gland juice and in centrifuged blood,

TABLE XI.

Changes in Weight after Single Doses of Tryparsamide.

Case No.	Name.	Dose tryparsamide.	Day of treatment.	1st week.	2nd week.	3rd week.	4th week	6th week.	8th week.	10th week.	12th week.	Total change.	Relapse.
		gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	
40	Esombo.....	0.5	51.0	49.5	51.5	51.0	51.0	51.0	51.5			+0.5	0†
50	Moangolo.....	0.5	29.5	29.5	30.0	30.5	30.5					+1.0	+
48	M'Bondo.....	1.0	50.0	51.0	51.0	51.5	52.0					+2.0	+‡
38	Daie.....	1.0	46.0	46.5	44.0	44.5	44.5	44.5				−1.5	0(?)
30	Mafuala.....	1.0	39.6	40.0	40.0	40.5	44.0	43.0	45.5	48.0		+8.4	0
28	Mago.....	1.0	33.0	33.0	32.5	33.5						+0.5	+
47	Mondeke.....	2.0	61.0	61.5	61.0	60.5						−0.5	+‡
43	Mambula.....	1.0*	27.0	26.5	26.5	25.5	26.0	27.5	27.5			+0.5	+†
46	Bokao.....	2.0	50.0	49.0	49.5	49.0	50.0	50.5	55.0			+5.0	0
5	Mondongo....	2.0	47.0	47.0	47.0	45.0	44.0	44.5	45.5	46.5	47.0	None.	0‖
36	Sudila.....	2.0	46.0	46.0	46.5	47.0	49.0	48.0				+2.0	+
45	K. Georges....	2.0	38.5	39.0	38.5	36.5	37.0	38.5	43.0			+4.5	0†
39	Mulamba.....	3.0*	55.5	56.5	57.0	58.5	57.5	58.0				+2.5	+‡
37	Bukasa.....	3.0	54.5	54.0	55.0	54.0	52.5	53.0	53.5	54.5		None.	0
41	Bajaka.....	3.0*	53.0	53.0	52.5	52.5	53.0	53.0				None.	+§
42	N'Keta.....	2.0*	36.0	35.0	33.5	34.5	34.0	34.5	36.0			None.	+**
51	Gwamuntele..	3.0	44.0	45.0	44.0	44.0	44.5	45.0	44.5			+0.5	0†
31	Lubaki.....	3.0	44.5	43.5	42.5	43.0	44.5	46.0	46.0	47.0		+2.5	0‡
68	Tuamene.....	5.0	62.5	62.0	61.5	62.5						None.	+
53	Ganzema.....	3.0	36.0	37.0	37.0	37.0						+1.0	+
73	Talamene.....	4.0	36.5	36.5	37.0	37.5	38.0					+1.5	0

* Intramuscular administration.

† Estivo-autumnal malaria.

‡ Filariasis.

§ Filariasis; malaria (?).

‖ Diffuse skin infection; filariasis; chronic diarrhoea.

** Craw-craw; intramuscular abscess.

cell counts of the spinal fluid, cell counts and hemoglobin estimations of the blood, and finally weight determinations—information of a fundamental character to be used in devising an effective system of

repeated dose therapy. In addition, other effects of treatment may be briefly considered.

Adenitis.—Polyadenitis is a constant symptom of human trypanosomiasis and of all of the superficial lymph glands those of the neck are most regularly involved. Palpation of these glands constitutes the general diagnostic method for the disease in the Belgian Congo, and enlarged cervical glands were present in all cases treated with tryparsamide except in 4 advanced patients who were being treated with other drugs. The glands most frequently hypertrophied

TABLE XII.

Increases in Weight of Non-Relapsed and Relapsed Patients after Single Doses of Tryparsamide.

Non-relapses.					Relapses.				
Case No.	Name.	Dose.	Total gain.	Time.	Case No.	Name.	Dose.	Total gain.	Time.
		mg. per kg.	*kg.*	*weeks*			*mg. per kg.*	*kg.*	*weeks*
40	Esombo.....	9	0.5	8†	50	Moangolo.....	17	1.0	4
30	Mafuala.....	25	8.4	10	48	M'Bondo.....	20	2.0	4*
46	Bokao.....	40	5.0	8	28	Mago.....	33	0.5	3
45	K. Georges....	52	4.5	8†	43	Mambula.....	37	0.5	8†
51	Gwamuntele...	67	0.5	8†	36	Sudila.....	44	2.0	6
31	Lubaki.....	68	2.5	10*	39	Mulamba.....	54	2.5	6*
73	Talamene.....	109	1.5	4	53	Ganzema.....	83	1.0	3
Average gain of 3.3 kg. in 4 to 10 weeks.					Average gain of 1.4 kg. in 3 to 8 weeks.				

* Filariasis.

† Estivo-autumnal malaria.

in our series were those of the posterior cervical triangle; in several cases a chain of enlarged glands were felt along one or both sides of the posterior portion of the neck, while the majority showed one or more enlarged glands at the base, either in the anterior or posterior cervical triangle. Usually the glands were about the size of a large pea or filbert, often slightly fusiform and of a peculiar soft but elastic consistency which has been compared to that of a ripe plum. In the cerebrospinal cases, the glands were usually smaller and firmer. Other superficial lymph glands are not commonly used as diagnostic signs.

Under treatment with single doses of tryparsamide, a marked reduction of the enlarged cervical glands was observed in all cases which did not relapse. They became much smaller and firmer and in certain patients whom we were able to follow for 2 or 3 months gland puncture was carried out with considerable difficulty. In relapsed patients, the condition of the glands was directly related to the duration of the peripheral sterilization; if this extended over 4 or 5 weeks, a distinct reduction was noticeable.

Temperature.—An irregular intermittent fever and rapid pulse are characteristic of human trypanosomiasis and during febrile periods the temperature is more often elevated in the afternoon than morning. Because one is obliged to take the temperatures in the axilla of the natives, correct readings are difficult to obtain and the readings are lower than they should be. The highest fever noted was usually around 38.5° C.; after treatment, a sharp drop occurred as shown in Charts I to III. The temperature charts of Bikoko #55 (Chart I) is interesting as it shows the prompt cessation of fever after the administration of the dose of only 0.3 gm. or 4.7 mg. per kilo; this patient had an unusually severe lymph gland infection but the dose was sufficient to produce a negative gland examination in 19 hours. Three other patients, M'Bondo #48, Yala #70, and Kakuma #65 (Chart I), show a similar drop in the temperature curve following the administration of 1.0, 3.0, and 4.0 gm. doses of tryparsamide. The charts of Esombo #40 (Chart II) and K. Georges #45 (Chart III) treated with single doses of 0.5 and 2.0 gm. cover periods of $6\frac{1}{2}$ and 9 weeks in which no trypanosomes were found in the lymph glands or blood. The intercurrent fever with increased pulse rate in these patients was due to an acute attack of estivo-autumnal malaria. K. Georges #45 was admitted during an afebrile period but it should be noted that his pulse rate was increased to 96 per minute.

Pulse.—The pulse rate in human trypanosomiasis is of considerable importance in diagnosis and in many of our cases it served as an index of the effect and duration of action of tryparsamide. The pulse itself is regular with a small volume and generally a lowered tension. The rate is usually disproportionately increased in relation to the fever and may persist above normal in afebrile periods.

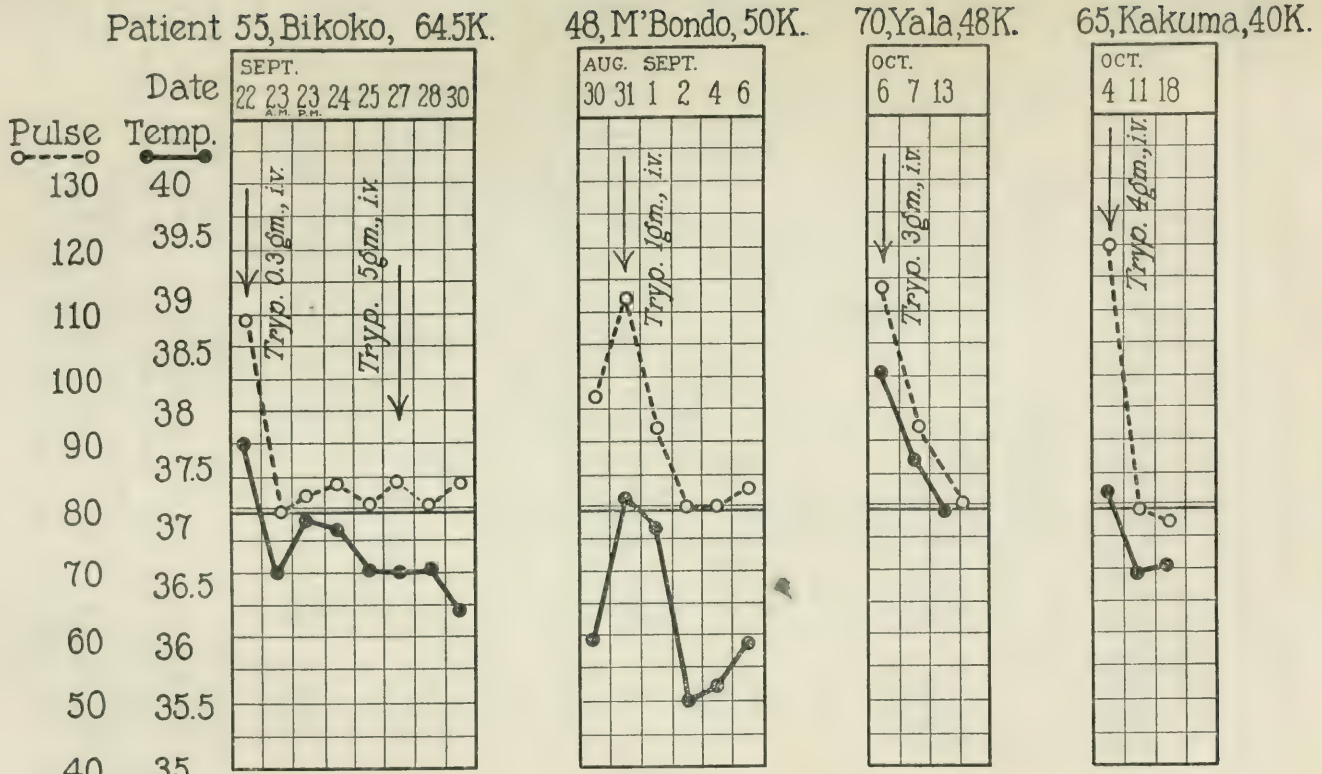

Patient 55, Bikoko, 64.5K.
48, M'Bondo, 50K.
70, Yala, 48K.
65, Kakuma, 40K.
Date
Pulse
Temp.
130
120
110
100
90
80
70
60
50
40
40
39.5
39
38.5
38
37.5
37
36.5
36
35.5
35
SEPT.
22 23 23 24 25 27 28 30
A.M. P.M.
Tryp. 0.3 gm., iv.
Tryp. 5 gm., iv.
AUG. SEPT.
30 31 1 2 4 6
Tryp. 1 gm., iv.
OCT.
6 7 13
Tryp. 3 gm., iv.
OCT.
4 11 18
Tryp. 4 gm., iv.

CHART I.

In the charts of the above 6 patients (Charts I to III), the effects of tryparsamide upon the pulse rate may be seen. Usually within 24 to 48 hours after treatment with a single dose, the pulse rate became normal or subnormal, unless some complicating infection or pathological condition existed—as for instance an acute or subacute malaria which was the case with Esombo #40 (Chart II) and K. Georges #45

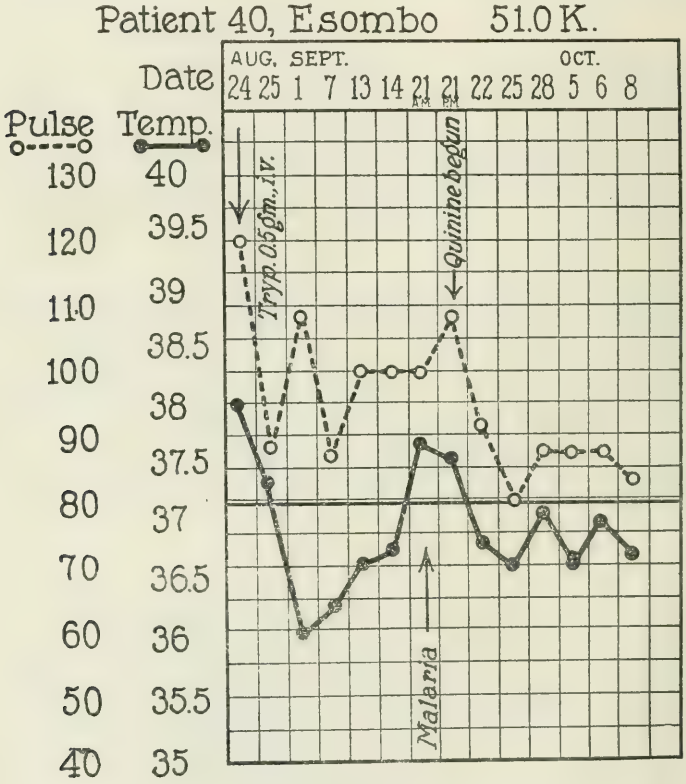


Chart II.

(Chart III). In a few patients in whom no complicating condition was found, the possibility of a still operative trypanosomal infection must be considered, although repeated microscopic examinations of lymph gland juice and centrifuged blood were negative. Such cases ultimately relapsed but the duration of peripheral sterilization varied widely. An increased pulse rate with slight or no fever during the

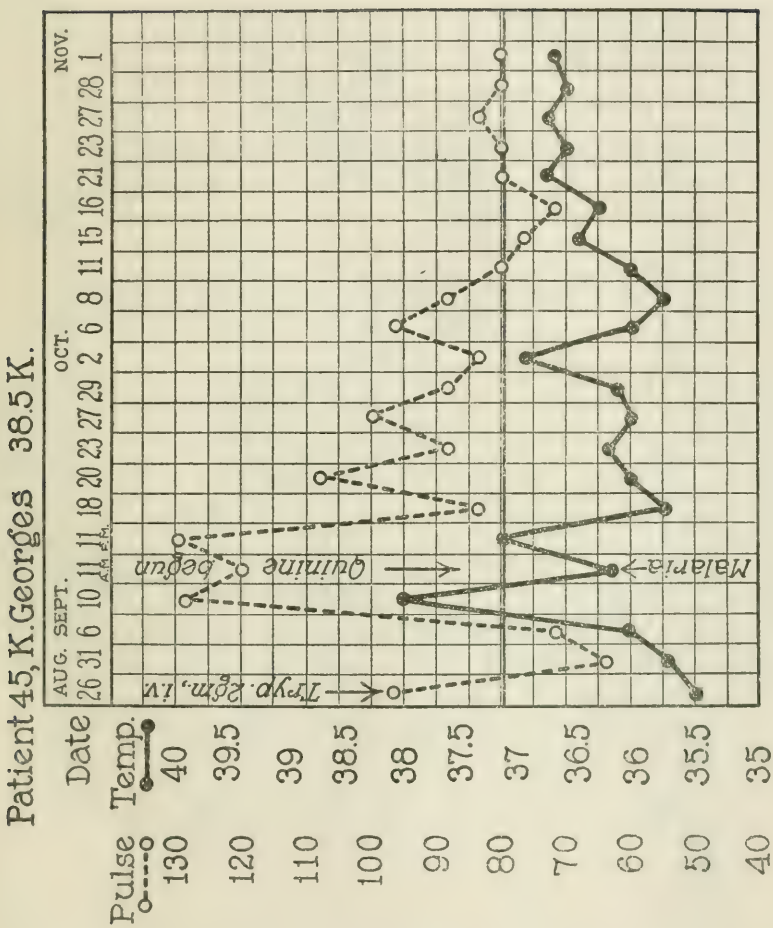

Patient 45, K. Georges 38.5 K.
Date
Temp.
Pulse
AUG. SEPT. OCT. NOV.
26 31 6 10 11 11 18 20 23 27 29 2 6 8 11 15 16 21 23 27 28 1
40 39.5 39 38.5 38 37.5 37 36.5 36 35.5 35
130 120 110 100 90 80 70 60 50 40
Tryp. 25m. i.v.
Quinine 0.25 begun
Malaria

CHART III.

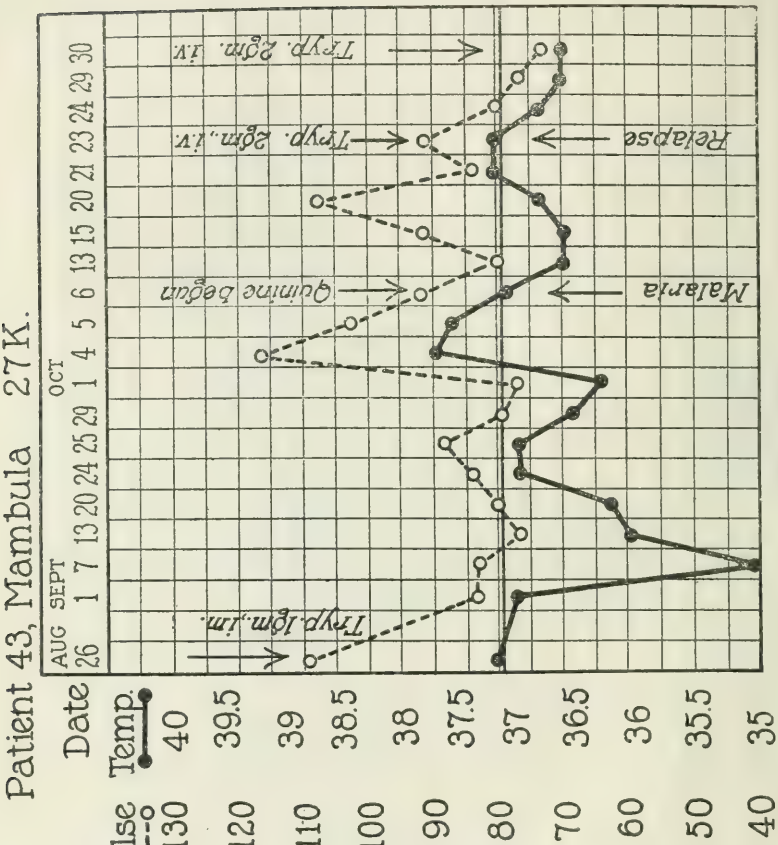

Patient 43, Mambula 27 K.
Date
Temp.
Pulse
AUG SEPT OCT
26 1 7 13 20 24 25 29 1 4 5 6 13 15 20 21 23 24 29 30
130 120 110 100 90 80 70 60 50 40
40 39.5 39 38.5 38 37.5 37 36.5 36 35.5 35
Tryp. 10m. im.
Quinine begun
Tryp. 20m. iv.
Relapse
Malaria
Tryp. 20m. iv.

CHART IV.

period of peripheral sterilization was found to possess considerable importance as an indication of a relapse, provided that other pathological factors were excluded as shown in Charts IV to VII.

Mambula #43 (Chart IV) after treatment on August 26, 1920, with 1.0 gm. of tryparsamide administered intramuscularly, showed a subnormal temperature and fairly normal pulse rate with negative

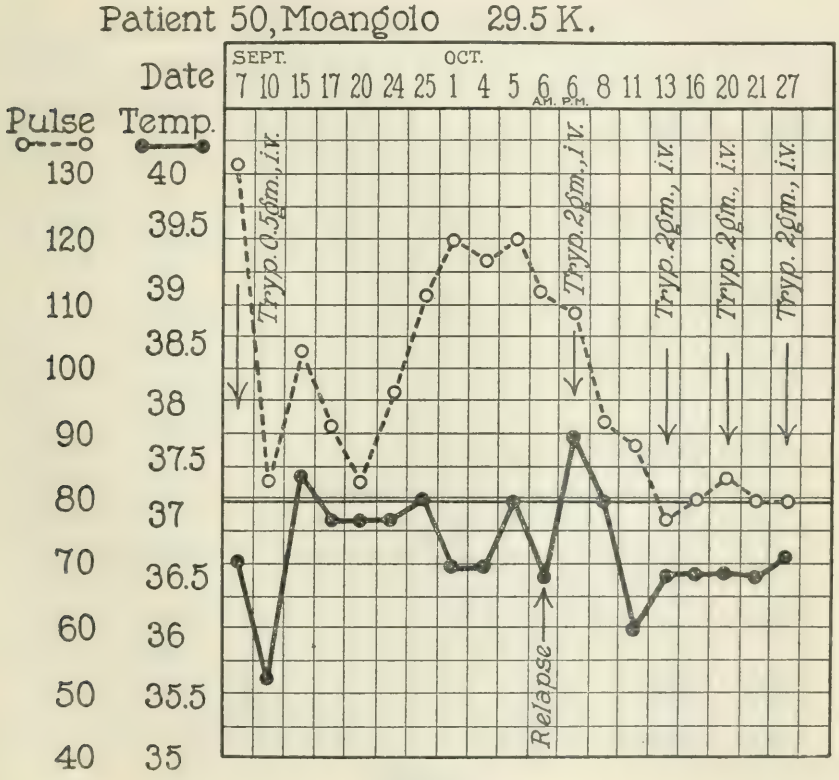


CHART V.

lymph gland and blood examinations until October 4, 1920, at which time he developed fever and a pulse rate of 116 per minute. Malarial parasites were found on October 6 and quinine therapy resulted in the return to a normal pulse rate until October 15, when an increase again occurred. During the following week, the pulse rate was irregularly increased without a proportionate rise in temperature and

trypanosomes were found on October 23. After retreatment, the pulse rate promptly fell to normal.

The chart of Moangolo #50 (Chart V) illustrates the type of case which leads one to suspect that the trypanosomal infection may still be active within the body although no organisms can be found in the lymph glands or blood. This boy was given 0.5 gm. of tryparsa-

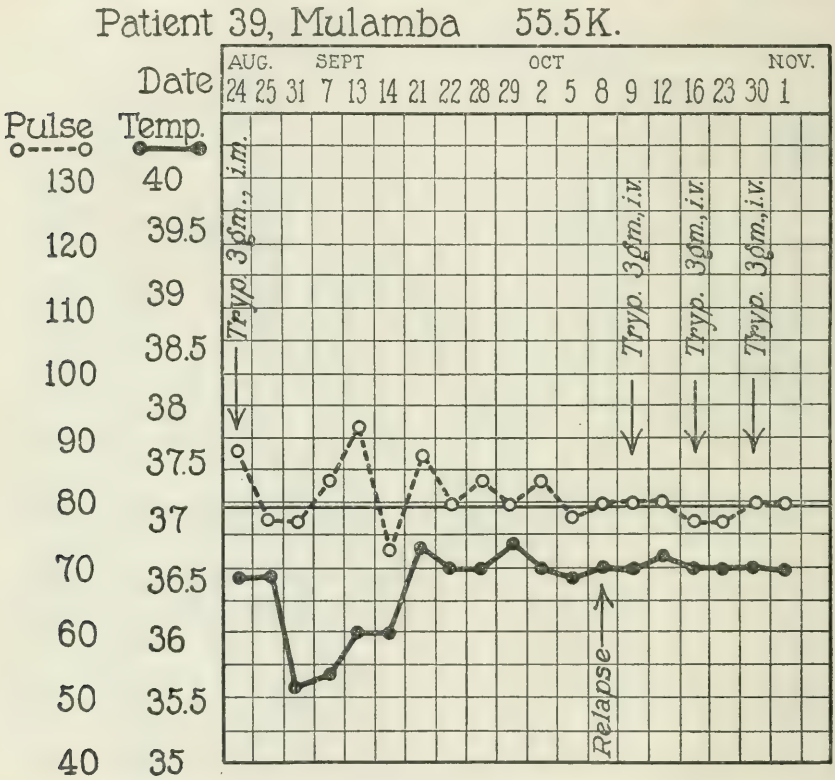


CHART VI.

mide on September 7. After an initial drop in pulse rate and temperature the pulse became disproportionately rapid, varying from 96 to 120 per minute for 16 days, and the temperature was somewhat irregular. No complicating infections could be found, however, and trypanosomes were not seen until October 6, 29 days after treatment. Retreatment had a very prompt effect upon both pulse and temperature as may be seen by referring to Chart V.

Charts VI and VII are illustrative of the less common type of reaction in which little or no increase in pulse rate takes place with a return of trypanosomes to the lymph glands or blood. Mulamba #39 (Chart VI) was treated August 24 with 3.0 gm. and during the following 6 weeks the pulse rate was irregular, varying from 72 to 92, but on the whole was not remarkable and no fever was observed.

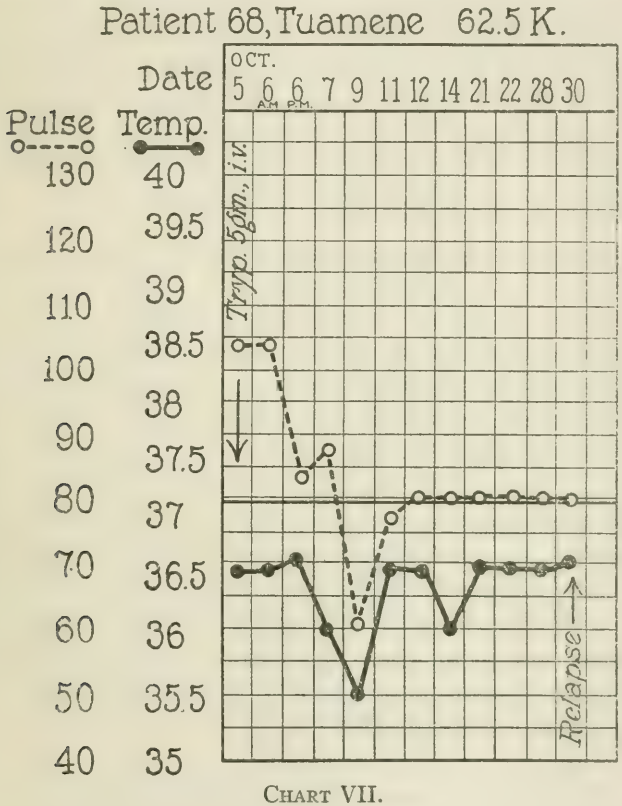


Chart VII.

Trypanosomes were found on October 8 and retreatment with tryparsamide caused a prompt cessation of pulse rate irregularity. A somewhat similar course of events is seen in the chart of Tuamene #68 (Chart VII), in whom a peripheral relapse was detected 25 days after treatment although the pulse rate had not been above normal for 23 days.

Symptoms.—Finally, as a result of the single dose treatment, a noticeable and continuous general improvement occurred in all patients in the first or beginning of the second stage of sleeping sickness leading them to wish to leave the lazaret or hospital or to cease coming to the laboratory. Conspicuous improvement was naturally most apparent in those patients whose ill appearance on admittance was most noticeable. The more definite symptoms of pains in the legs, arms, and joints, headaches, weakness, and general malaise disappeared during the first week after treatment and did not return even in those cases which eventually relapsed, presumably because retreatment was immediately instituted. Within 1 or 2 weeks, all the patients in this group appeared well and it was in many instances only with the greatest difficulty that further visits were secured.

ADMINISTRATION OF REPEATED DOSES IN EARLY CASES.

The consensus of opinion is that human trypanosomiasis can best be influenced by a system of repeated doses of the drugs commonly employed and several courses of atoxyl alone or combined with tartar emetic are recommended both in early and late cases. Treatment with repeated doses of tryparsamide given intravenously was regularly carried out as soon as the duration of action of single doses was determined in a number of instances. The patients thus treated fall into two main groups according to the period of the disease as determined by the spinal fluid. The first group consisted of patients in the first stage of the disease or in the beginning of the second period and included those who suffered a relapse after single doses as well as a number of previously untreated cases and the observations carried out on this group will be considered first.

Early Cases Previously Treated with One Dose.

This group consists first of 12 patients treated with a single dose of tryparsamide of from 17 to 83 mg. per kilo, who showed a return of trypanosomes in the blood and lymph glands after periods of peripheral sterilization varying from 17 to 58 days, and were retreated with repeated doses of the drug given at weekly or fortnightly intervals as shown in Table XIII. 2 other patients are also considered in this

TABLE XIII.

Treatment of Relapsed Early Cases with Repeated Doses of Tryparsamide. Weekly Administration.

Case No.	Name.	Original weight.	No. of cells in spinal fluid per cmm. before treatment.	Doses of tryparsamide adm. i. v.	Remarks.
		kg.		*gm.*	
28	Mago.....	33	5 (1 l.m.)	1.0 (33 mg. per kg.) Aug. 16 2.0 Sept. 4, 11, 18, 25 Total 9.0 gm.	L. P. Oct. 16 Normal. 2.5 lymph.
50	Moangolo..	29.5	Normal.	0.5 (17 mg. per kg.) Sept. 7 2.0 Oct. 6, 13, 20, 27 Total 8.5 gm.	L. P. Oct. 16 1.2 lymph. 1 l.m.
39	Mulamba..	55.5	4	3.0 (54 mg. per kg.) Aug. 24* 3.0 Oct. 9, 16, 23, 30 Total 15.0 gm.	L. P. Sept. 28 Normal.
36	Sudila.....	46	20	2.0 (44 mg. per kg.) Aug. 19 3.0 Oct. 9 2.0 Oct. 16, 23, 30 2.0 to be given Nov. 6, 13, 20 Total 17.0 gm.	L. P. Sept. 28 Normal. 2.5 lymph.
42	N'Keta....	36	10	2.0 (57 mg. per kg.) Aug. 26* 2.0 Oct. 16, 23, 30 2.0 to be given Nov. 6, 13 Total 12.0 gm.	L. P. Sept. 29 Normal. 0.6 lymph.
43	Mambula..	27	7.5	1.0 (37 mg. per kg.) Aug. 26* 2.0 Oct. 23, 30 2.0 to be given Nov. 6, 13, 20 Total 11.0 gm.	L. P. Sept. 29 Normal. 2.1 lymph.
53	Ganzema...	36	3.4 (1 l.m.)	3.0 (83 mg. per kg.) Sept. 13 4.0 Oct. 4, 18, 25 4.0 to be given Nov. 4 Total 19.0 gm.	
68	Tuamene...	62.5	5 (1 l.m.)	5.0 (80 mg. per kg.) Oct. 5 5.0 Nov. 1 3.0 to be given Nov. 8, 15, 23, 30 Total 22.0 gm.	

TABLE XIII—*Concluded.*

Case No.	Name.	Original weight.	No. of cells in spinal fluid per cmm. before treatment.	Doses of tryparsamide adm. i. v.	Remarks.
		kg.		*gm.*	
63	N'Kaka....	42.5	Normal.	2.0 (47 mg. per kg.) Oct. 4 3.0 Oct. 21. Did not return Oct. 28 3.0 to be given weekly for 5 inj. Total 20.0 gm.	
38	Daie.....	46	(?) R. B. C.	1.0 (22 mg. per kg.) Aug. 21 3.0 Sept. 30 1.0 Oct. 7, 14, 21, 28 1.0 to be given Nov. 4, 11, 18, 25 Total 12.0 gm.	L. P. Sept. 27 35 lymph. 1 l.m.
				Fortnightly Administration.	
48	M'Bondo..	50	5.0	1.0 (20 mg. per kg.) Aug. 31 2.5 Sept. 24, Oct. 8, 22 2.5 to be given Nov. 5 Total 11.0 gm.	L. P. Oct. 6 Normal. 1 lymph.
47	Mondeke...	61	7.5	2.0 (33 mg. per kg.) Aug. 28 4.0 Sept. 24, Oct. 8, 22 4.0 to be given Nov. 5 Total 18.0 gm.	L. P. Oct. 6 Normal. 3 lymph.
41	Bajaka.....	53	15.0	3.0 (57 mg. per kg.) Aug. 26* 4.0 Oct. 2, 16, 30 4.0 to be given Nov. 13 Total 19.0 gm.	L. P. Sept. 30 7.5 lymph. 2 l. m.
55	Bikoko.....	64.5	Normal.	0.3 (4.7 mg. per kg.) Sept. 22 5.0 Sept. 27, Oct. 11, 25 5.0 to be given Nov. 5 Total 20.3 gm.	

* Intramuscular administration.

section: Bikoko #55, who was given the small dose of 0.3 gm. (4.7 mg. per kilo) which produced a peripheral sterilization for 5 days and Daie #38, whose second spinal fluid examination showed an increased cell content, although no trypanosomes were detected in the lymph

glands, blood, or spinal fluid. 10 of these patients were treated at weekly intervals with 4 to 9 injections of doses varying from 20 to 111 mg. per kilo, while 4 were placed on a fortnightly schedule. The drug was given intravenously in all instances.

The observations at present available may be considered briefly. In 8 patients who could be examined shortly after the administration of the first dose, no trypanosomes were found in the lymph glands or centrifuged blood at 5, 6, 7, 8, and 15 hours after treatment, showing that the initial effect of the drug in retreatment is similar to that observed in previously untreated cases. Several examinations for trypanosomes carried out on all the patients during the course of retreatment were uniformly negative. In one case, Mago #28, the course of 4 doses of 2.0 gm. given at weekly intervals, was completed so that subsequent observation can be reported here. During the following 6 weeks, examinations of lymph glands and blood were negative and in addition a lumbar puncture done 61 days after the first single dose showed a normal spinal fluid—2.5 lymphocytes per cmm.—whereas the original count was 5 lymphocytes per cmm. with 1 large mononuclear in the preparation (Table XIV).

The spinal fluid of 3 other patients, Moangolo #50, M'Bondo #48, and Mondeke #47, examined 10 and 12 days after the beginning of the course of repeated doses and approximately 5 weeks after the initial single dose, showed that the disease had not progressed to the second stage and in 2 patients the slightly increased cell counts had become normal (Table XIV).

Thus in these 4 instances the administration of 2 to 5 small or medium sized doses of tryparsamide, though widely spaced apart and covering a period in which a peripheral relapse occurred, had an apparent effect in arresting further cerebrospinal involvement.

Effect on Blood Count.—The blood was studied in 10 patients retreated with repeated doses at weekly or fortnightly intervals following a relapse after a previous single dose. The first blood examination was made before the initial single dose while the second occurred about 2 months later during or at the end of the course of repeated doses, as shown in Table XV. A marked secondary anemia was the outstanding abnormality of the blood count on admittance.

For convenience of comparison, the changes noted in the red and white cell counts and hemoglobin estimation have been tabulated separately (Tables XVI to XVIII). It was found that after treatment with tryparsamide, an increase in the number of red cells of from 3 to 47 per cent occurred in 8 patients. The most marked improvement was usually observed in those patients who had the lowest original counts. In 2 patients, Moangolo #50 and Bikoko #55, the first count was practically normal and the second count made 50 and 33 days

TABLE XIV.

Changes in Cell Count, Spinal Fluid of Relapsed Cases During Retreatment with Repeated Doses of Tryparsamide.

Case No.	Name.	Initial weight.	Doses adm. i. v.	Cell count spinal fluid initial L.P.	Cell count spinal fluid 2nd L. P.	Interval between L. P.
		kg.	*gm.*			*days*
28	Mago.....	33	1 x 1.0 9.0 4 x 2.0 in 40 days.	5 (1 l.m.)	Normal.	61
50	Moangolo.....	29.5	1 x 0.5 4.5 2 x 2.0 in 36 days.	Normal.	1.2 lymph. 1 l.m.	39
48	M'Bondo.....	50	1 x 1.0 3.5 1 x 2.5 in 25 days.	5	Normal.	37
47	Mondeke.....	61	1 x 2.0 6.0 1 x 4.0 in 27 days.	7.5	Normal.	39

after the administration of the single dose of tryparsamide showed a slight decrease of 5 and 8 per cent respectively.

The improvement in hemoglobin content of the blood was even more striking as may be seen in Table XX. Each patient showed a gain ranging from 7 to 71 per cent. As was the case after the administration of single doses, the most marked increase occurred in those patients whose hemoglobin was below 60 per cent before treatment, while the two patients, Moangolo #50 and Bikoko #55, with normal red cell counts showed an improvement of 45 and 7 per cent respectively.

TABLE XV.

Changes in Blood Count of Relapsed Early Cases after Retreatment.

Case No	Name.	Total No. of doses adm. i. v.	1st blood count.	2nd blood count.	Changes.	Time after 1st treatment of 2nd B. C.
		gm.			*per cent*	*days*
28	Mago.....	1 x 1.0	R. B. C. 4,320,000	4,800,000	+ 11	68
	33 kg.	4 x 2.0	Hb. 55 %	70 %	+ 27	
			W. B. C. 5,700	8,000	+ 40	
50	Moangolo.....	1 x 0.5	R. B. C. 5,056,000	4,800,000	− 5	50
	29.5 kg.	3 x 2.0	Hb. 55 %	80 %	+ 45	
			W. B. C. 11,200	10,000	− 11	
39	Mulamba.....	1 x 3.0*	R. B. C. 3,840,000	4,000,000	+ 4	59
	55.5 kg.	2 x 3.0	Hb. 52 %	80 %	+ 54	
			W. B. C. 12,800	7,000	− 45	
36	Sudila.....	1 x 2.0	R. B. C. 4,160,000	5,120,000	+ 23	65†
	46 kg.	1 x 3.0	Hb. 60 %	65 %	+ 8	
		1 x 2.0	W. B. C. 8,600	8,000	− 7	
42	N'Keta.....	1 x 2.0*	R. B. C. 3,200,000	4,000,000	+ 25	65‡
	36 kg.	2 x 2.0	Hb. 35 %	60 %	+ 71	
			W. B. C. 4,000	8,000	+100	
38	Daie.....	1 x 1.0	R. B. C. 3,720,000	4,000,000	+ 8	67
	46 kg.	1 x 3.0	Hb. 60 %	70 %	+ 17	
		3 x 1.0	W. B. C. 7,000	6,000	− 14	
48	M'Bondo.....	1 x 1.0	R. B. C. 3,200,000	3,680,000	+ 15	53§
	50 kg.	2 x 2.5	Hb. 50 %	65 %	+ 30	
			W. B. C. 9,200	8,000	− 13	
47	Mondeke.....	1 x 2.0	R. B. C. 4,480,000	4,640,000	+ 3	55§
	61 kg.	2 x 4.0	Hb. 65 %	75 %	+ 15	
			W. B. C. 8,400	8,000	− 5	
41	Bajaka.....	1 x 3.0*	R. B. C. 3,044,000	4,480,000	+ 47	60**
	53 kg.	2 x 4.0	Hb. 45 %	75 %	+ 67	
			W. B. C. 4,800	9,000	+ 88	
55	Bikoko.....	1 x 0.3	R. B. C. 5,240,000	4,830,000	− 8	33‖
	64.5 kg.	3 x 5.0	Hb. 75 %	80 %	+ 7	
			W. B. C. 8,000	8,000	—	

* Intramuscular administration.

† Malaria (?).

‡ Intramuscular abscess; craw-craw.

§ Filariasis.

‖ Malaria.

** Filariasis; malaria (?).

TABLE XVI.

Changes in Red Blood Cell Counts of Patients Previously Treated with Single Doses—Retreated with Repeated Doses.

Case No.	Name.	Total amount tryparsamide.	Time between 1st and 2nd R. B. C.	1st R. B. C. before treatment.	R. B. C. after treatment.	Change.
		mg. per kg.	*days*			*per cent*
41	Bajaka	209	60	3,044,000	4,480,000	+47
42	N'Keta	171	65	3,200,000	4,000,000	+25
48	M'Bondo	120	53	3,200,000	3,680,000	+15
38	Daie	154	67	3,720,000	4,000,000	+ 8
39	Mulamba	162	59	3,840,000	4,000,000	+ 4
36	Sudila	154	65	4,160,000	5,120,000	+23
28	Mago	297	68	4,320,000	4,800,000	+11
47	Mondeke	165	55	4,480,000	4,640,000	+ 3
50	Moangolo	201	50	5,056,000	4,800,000	− 5
55	Bikoko	244.7	33	5,240,000	4,830,000	− 8

TABLE XVII.

Changes in Hemoglobin Content of Patients Previously Treated with Single Doses—Retreated with Repeated Doses.

Case No.	Name.	Total amount tryparsamide.	Interval between 1st and 2nd hemoglobin examinations.	1st hemoglobin.	2nd hemoglobin.	Increase.
		mg. per kg.	*days*			*per cent*
42	N'Keta	171	65	35	60	+71
41	Bajaka	209	60	45	75	+67
48	M'Bondo	120	53	50	65	+30
39	Mulamba	162	59	52	80	+54
50	Moangolo	201	50	55	80	+45
28	Mago	297	68	55	70	+27
38	Daie	154	67	60	70	+17
36	Sudila	154	65	60	65	+ 8
47	Mondeke	165	55	65	75	+15
55	Bikoko	244.7	33	75	80	+ 7

The white cells in practically all cases were within normal limits after retreatment as shown in Table XVIII. 3 patients, N'Keta #42, Bajaka #41, and Mago #28, who originally had a leukopenia, showed an increase in the number of white cells of 100, 88, and 40 per cent respectively—in the others, the white cells remained or approached normal.

These observations are in accord with those noted after single doses and indicate that the blood picture of patients suffering with trypanosomiasis tends to return to normal limits after treatment with tryparsamide.

Effect upon the Weight.—The changes in weight of 12 patients during the period of peripheral sterilization and subsequently after retreatment with repeated doses of tryparsamide, are shown in Table XIX. The majority of observations extend over a period of 10 weeks during which time from 2 to 6 doses were given. Doses of 2.0, 3.0, and 4.0 gm. were used in most instances.

TABLE XVIII.

Changes in White Blood Cell Counts of Patients Previously Treated with Single Doses—Retreated with Repeated Doses.

Case No.	Name.	Total amount tryparsamide.	Interval between 1st and 2nd W. B. C.	1st W. B. C. before treatment.	2nd W. B. C. after treatment.	Change.
		mg. per kg.	*days*			*per cent*
42	N'Keta	171	65	4,000	8,000	+100
41	Bajaka	209	60	4,800	9,000	+ 88
28	Mago	297	68	5,700	8,000	+ 40
38	Daie	154	67	7,000	6,000	− 14
55	Bikoko	244.7	33	8,000	8,000	−
47	Mondeke	165	55	8,400	8,000	− 5
36	Sudila	154	65	8,600	8,000	− 7
48	M'Bondo	120	53	9,200	8,000	− 13
50	Moangolo	201	50	11,200	10,000	− 11
39	Mulamba	162	59	12,800	7,000	− 45

The effect of tryparsamide treatment upon the weight of these patients was as follows: 6 patients gained from 1.5 to 3.5 kilos, 4 showed no change, and 2 showed a loss of 1.5 kilos. Concurrrent infections were present in 5 of the 6 patients who did not gain, while in the case of Daie #38 the explanation is not entirely clear.

In general, it may be said that the physical improvement of these patients which occurred after treatment with single doses of tryparsamide continued during the course of repeated doses as shown by an increase of weight in uncomplicated cases and by the beneficial effect upon the blood picture.

TABLE XIX.

Changes in Weight of Relapsed Early Cases after Retreatment with Repeated Doses of Tryparsamide.

Case No.	Name.	Total no. of doses.	Original weight.	2nd week.	4th week.	6th week.	8th week.	10th week.	Total change.
		gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
28	Mago.....	1 x 1.0 4 x 2.0	33.0	32.5	33.0	34.5	33.5	34.5	+1.5
50	Moangolo.....	1 x 0.5 3 x 2.0	29.5	30.0	30.5	31.5	31.5		+2.0
39	Mulamba.....	1 x 3.0* 3 x 3.0	55.5	57.0	57.5	58.0	58.5	59.0	+3.5‖
36	Sudila.....	1 x 2.0 1 x 3.0 2 x 2.0	46.0	46.5	49.0	48.0	47.5	46.0	0†
42	N'Keta.....	1 x 2.0* 2 x 2.0	36.0	33.5	34.0	34.5	35.5	36.0	0§
43	Mambula.....	1 x 1.0* 1 x 2.0	27.0	26.5	26.0	27.5	27.5	27.0	0‡
53	Ganzema.....	1 x 3.0 3 x 4.0	36.0	37.0	37.0	37.0	37.5		+1.5
38	Daie.....	1 x 1.0 1 x 3.0 4 x 1.0	46.0	44.0	44.5	44.5	44.5	44.5	−1.5
48	M'Bondo.....	1 x 1.0 3 x 2.5	50.0	51.0	52.0	52.0	52.5	53.5	+3.5‖
47	Mondeke.....	1 x 2.0 3 x 4.0	61.0	61.0	60.5	61.0	61.0	61.0	0‖
41	Bajaka.....	1 x 3.0* 2 x 4.0	53.0	52.5	53.0	53.0	53.0	51.5	−1.5**
55	Bikoko.....	1 x 0.3 3 x 5.0	64.5	63.5	65.0	66.0			+1.5‡

* Intramuscular administration.

† Malaria (?).

‡ Malaria.

§ Intramuscular abscess; craw-craw.

‖ Filariasis.

** Filariasis; malaria (?).

Previously Untreated Early Cases.

13 previously untreated patients in the first period of trypanosomiasis were treated with repeated doses of from 1.0 to 5.0 gm. of tryparsamide given at weekly and fortnightly intervals (Tables XX

TABLE XX.

Repeated Dose Treatment of Early Previously Untreated Cases. Weekly Administration.

Case No.	Name.	Weight.	No. cells spinal fluid per cmm.	Doses of tryparsamide adm. i. v.	Total.
		kg.			*gm.*
72	N'Ganzobo..	26	Normal.	1.0 gm. (38 mg. per kg.) Oct. 6, 13, 20, 27 1.0 " to be given on Nov. 3, 10	6.0
78	Itoma.....	52		1.0 " (19 mg. per kg.) Oct. 29 2.0 " (38 " " ") Nov. 4, 11, 18, 25, Dec. 2, 9, 16	15.0
71	Madeele....	24	Normal.	1.0 " (42 " " ") Oct. 6, 13, 20, 27	4.0
64	Dano.....	40	0.6	2.0 " (50 " " ") Oct. 4, 21, 28 2.0 " to be given Nov. 4, 11	10.0
69	Sabole.....	67.5	7.5 1 l.m.	3.0 " (44 mg. per kg.) Oct. 5, 12, 19, 26 Nov. 2	15.0
77	V. Makau...	57.0	3.8 1 l.m.	1.0 " (18 " " ") Oct. 28 3.0 " (54 " " ") Oct. 29 3.0 " to be given Nov. 5, 12, 19, 26	16.0
58	Kaniki.....	42.5	1.2	2.5 " (58 mg. per kg.) Sept. 28, Oct. 7, 14, 21, 28 2.5 " to be given Nov. 4	15.0
70	Yala.....	48.0	8.0	3.0 " (63 mg. per kg.) Oct. 6, 13, 20, 27	12.0
62	Sawa.....	57.0	5.3	4.0 " (70 " " ") Oct. 4, 12, 19 4.0 " to be given Nov. 3	16.0

and XXI). Trypanosomes were found in lymph gland juice before treatment in all instances; spinal fluid examination showed a normal cell count in 6 cases and a slight increase of cells in 5; lumbar puncture was not done in 2 cases.

The observations of this group at present available were made during the first month of treatment and are summarized as follows: The preliminary sterilization of lymph glands and blood together with a sharp drop in the accelerated pulse rate and fever occurred as previously described and in those patients under observation for 3 or 4 weeks a decrease in size and induration of the cervical lymph glands was observed; subsequent examinations for trypanosomes in each patient made at various times were uniformly negative. With the

TABLE XXI.

Repeated Dose Treatment of Early Previously Untreated Cases. Fortnightly Administration.

Case No.	Name.	Weight.	No. cells spinal fluid per cmm.	Doses of tryparsamide adm. i. v.	Total.
		kg.			*gm.*
56	Yaya.....	49.5	1	2.0 gm. (40 mg. per kg.) Sept. 24, Oct. 8, 25 2.0 " to be given Nov. 8, 22	10
65	Kakuma.....	40.0	4	4.0 " (100 mg. per kg.) Oct. 4, 18, Nov. 1 4.0 " to be given Nov. 15, 29	20
67	M'Bwongo...	27.0		3.0 " (111 mg. per kg.) Oct. 4, 18 Nov. 1 3.0 " to be given Nov. 15, 29	15
66	Popa.....	42.5	2.5	5.0 " (118 mg. per kg.) Oct. 4, 18 5.0 " to be given Nov. 3, 15	20

exception of Kaniki #58, who had syphilis, and Yaya #56, who had malaria, there was prompt cessation of the pains, weakness, and general malaise complained of on admittance.

The changes in weight of 11 patients are given in Table XXII; 6 patients gained from 0.5 to 2.5 kilos, 1 lost 2 kilos from no apparent cause, while 4 showed no change. It was not possible to carry out a detailed study of this group and blood counts of only 2 patients were made; these, however, showed a marked improvement during the period of treatment comparable to that previously described.

This group of patients has been included in the present paper to illustrate the application of a preliminary system of repeated dose therapy based upon the information obtained from the use of single doses. It is probable that various modifications will be made in the future; but just as it was necessary to determine the effect of single doses before proceeding to the use of repeated doses, so it is imperative to study the results of a few repeated doses in a certain number of cases before attempting a more complicated method of therapy. Further observations on these patients will be reported later.

TABLE XXII.

Changes in Weight of Early Cases Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	Doses.	Initial weight.	1st week.	2nd week.	3rd week.	4th week.	Total change.
		gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
72	N'Ganzobo.....	3 x 1.0	26	27	27.5	27.5		+1.5
71	Madeele.....	4 x 1.0	24	24.5	24.5	24.5		+0.5
64	Dano.....	2 x 2.0	40		40.0	38.0		−2.0
58	Kaniki.....	5 x 2.5	42.5	42.5	44.0	44.5	45.0	+2.5*
70	Yala.....	4 x 3.0	48	48.5	49.0	49.0	49.5	+1.5
62	Sawa.....	3 x 4.0	57	57.8	58.0			+1.0
69	Sabole.....	4 x 3.0	67.5	66.0	66.5	67.0	67.5	0
56	Yaya.....	3 x 2.0	49.5	49.5	48.5	49.0	49.5	0†
65	Kakuma.....	2 x 4.0	40.0		40.0			0
67	M'Bwonga.....	2 x 3.0	27.0	27.5	27.5	27.5	28.0	+1.0
66	Popa.....	2 x 5.0	42.5	43.0	42.5			0

* Syphilis.

† Malaria.

ADMINISTRATION OF REPEATED DOSES IN ADVANCED CASES.

35 patients whose spinal fluid showed that the disease had involved the cerebrospinal system represent the second group treated with repeated doses of tryparsamide given intravenously. Of these, 2 patients deserted after the first injection of the drug, 2 were practically moribund on admittance, and 3 others were under observation for such a short time that their records are not included. The remaining 28 patients may be conveniently considered in two subdivisions: 18 who had not previously been treated so far as known and 10 who had been treated before. The spinal fluids of the patients examined before

the administration of tryparsamide showed from 15 to 572 lymphocytes per cmm., and large mononuclears and *cellules en mûre* in most instances, while most of the patients suffered from pronounced nervous and mental symptoms and were anemic and debilitated. Trypanosomes were found in the cervical lymph nodes of 21 patients; they were not found in 7 patients who were being treated with atoxyl and tartar emetic at the time of transfer to tryparsamide therapy. Certain outstanding effects of the tryparsamide treatment as measured by changes in the spinal fluid, the blood, and body weight will be considered in the following sections and the size and number of doses of tryparsamide given to each patient will be described in connection with these observations.

Previously Untreated Advanced Cases.

1. Course of Treatment.

Effect upon the Spinal Fluid.—The most conspicuous concrete effect of the treatment of advanced cases of trypanosomiasis with tryparsamide was the marked and rapid decrease in the cell content of the spinal fluid accompanied by a definite clinical improvement. The cell changes observed in 7 previously untreated, advanced patients are shown in Table XXIII. The first examination before treatment showed an increase of lymphocytes in the spinal fluid varying from 17.5 to 285 per cmm. and in addition large mononuclear and mulberry cells were seen in 4 instances, indicating a considerable degree of cerebrospinal involvement. The diagnosis of trypanosomiasis was made by finding the organisms in the cervical lymph glands of all 7 patients; negative examinations followed the first treatment as previously described.

The system of therapy employed in these patients consisted of 3 to 9 intravenous injections of tryparsamide of from 1.0 to 5.0 gm. doses usually at weekly or semi-weekly intervals over an average period of 30 days. The following abstracts indicate the outstanding features of individual cases.

Loyoko № 18.—Soldier on sick leave from a "virulent" region. Good state of nutrition but very anemic. Mental condition normal. Marked filariasis. 60 kilos. General glandular enlargement. Gland puncture ++. Spinal fluid 17.5 lympho-

TABLE XXIII.

Cell Changes of Spinal Fluid in Advanced Cases Treated with Repeated Doses of Tryparsamide.
(No Previous Treatment.)

Case No.	Name.	Course of tryparsamide. Total.	Cell content spinal fluid before treatment.	Cell content spinal fluid after treatment. Per cent change.	3rd L. P. after treatment. Per cent change.	Remarks.
18	Loyoko..... 60 kg.	3 x 3.0 gm. 9.0 gm. in 28 days. 150 mg. per kg.	17.5	3.4 (35 days) 80%	2.5 (82 days) 1 l.m.	Marked improvement.
49	Makalamba... 52.5 kg.	9 x 1.0 gm. 9.0 gm. in 29 days. 171 mg. per kg.	22.0	4.2 (38 days) 80%		Marked improvement.*
9	Bolendo..... 49.0 kg.	5 x 2.0 gm. 10.0 gm. in 34 days. 204 mg. per kg.	47.5	10 (39 days) 1 l.m. 70%		Marked improvement.*
44	N'Kusu..... 38.5 kg.	4 x 4.0 gm. 16.0 gm. in 29 days. 416 mg. per kg.	47.5 l.m.+	15 (32 days) 1 l.m. 68%	22 (55 days)	Improvement.*
32	N'Siola..... 40 kg.	4 x 4.0 gm. 16.0 gm. in 32 days. 400 mg. per kg.	52.5 l.m.+	15 (36 days) 70%	3.1 (66 days)	Marked improvement.
29	Fulu..... 25.5 kg.	8 x 1.0 gm. 8.0 gm. in 33 days. 314 mg. per kg.	142.5 l.m.+	22.5 (36 days) 84%		Marked improvement.*
35	Koloshi..... 44.5 kg.	4 x 5.0 gm. 20.0 gm. in 29 days. 449 mg. per kg.	285.0 l.m.+ m.+	12.5 (35 days) 1 l.m. 96%	8 (64 days) 1 l.m.	Marked improvement.

l.m.—Large mononuclears.
m.—Mulberry cells.
* Tryparsamide treatment resumed.

cytes per cmm. He was given 3 doses of 3.0 gm. (150 mg. per kilo) at intervals of 2 weeks or a total of 9.0 gm.; 5 weeks after the beginning of treatment, the cell count had fallen to 3.4 per cmm., a decrease of 80 per cent. As his general condition had materially improved, he returned to his military duties and during the following 2 months he continued to work each day. A third lumbar puncture done 12 weeks after the first dose of tryparsamide showed a cell content of 2.5 lymphocytes per cmm. with, however, 1 large mononuclear; at this time, a few tiny hard cervical lymph glands could be palpated; his blood count had materially improved. No further treatment is to be given this patient unless trypanosomes are found in the blood or lymph glands or unless there is an increase in the cells of the spinal fluid.

Makalamba # 49.—Fisherman. Good physical and mental condition; had not stopped work. 52.5 kilos. General glandular enlargement. Gland puncture +. Spinal fluid 22 lymphocytes per cmm. He was treated with 9 doses of 1.0 gm. each (19 mg. per kilo) administered twice a week, or a total of 9.0 gm. in 29 days. The second lumbar puncture done 38 days after the first showed a cell content of 4.2 lymphocytes per cmm. or a decrease of 80 per cent. After the course of tryparsamide was finished, he wished to return home but as the duration of the effect of his treatment was not known, a second course was begun. Despite an acute attack of malaria which developed under observation and the detection of a positive Wassermann reaction, he remained in excellent physical condition. There was a marked regression of the cervical adenitis.

Bolendo # 9.—Wife of a soldier. Well nourished but anemic and weak and had been unable to work. Mental condition normal. 49 kilos. General glandular enlargement. Gland puncture ++. Spinal fluid 47.5 lymphocytes per cmm. She was given 5 doses of 2.0 gm. in 34 days—a total of 10.0 gm. or 204 mg. per kilo. 39 days after the first dose of tryparsamide, her spinal fluid contained 10 lymphocytes per cmm., a decrease of 70 per cent, with 1 large mononuclear seen in the counting chamber. After a month's intermission, a second course of 3 doses of 4.0 gm. each was given in 18 days; a subsequent lumbar puncture was refused. There was a noticeable physical improvement, with a marked gain in weight during the first course of treatment which continued during the period of observation. The cervical lymph glands became very small and indurated.

N'Kusu # 44.—Laborer from a "virulent" region. Undernourished and anemic; unable to work. Uncertain mental state. 38.5 kilos. Typical chains of enlarged lymph glands on both sides of neck. Gland puncture ++. Spinal fluid 47.5 lymphocytes per cmm.; large mononuclears +. He was given 4 doses of 4.0 gm. of tryparsamide at 14, 7, and 8 day intervals or 416 mg. per kilo in 29 days, with no untoward effects. A second lumbar puncture, 32 days after the first, showed a cell content of 15 lymphocytes with 1 mononuclear, a decrease of 68 per cent. A third examination, 55 days after admittance, showed 22 lymphocytes per cmm. indicating that the effect of the drug had not continued to produce a further diminution of cells but on the other hand had sufficed to prevent a return to the original condition. A second course of tryparsamide was begun. Physically,

there was a moderate improvement during the period of observation and he wished to return home; the cervical glands decreased markedly in size and became very indurated; mentally, there was definite improvement to a normal status.

N'Siola # 32.—Workman; suspected case. Good state of nutrition. Mental condition normal. 40 kilos. General glandular enlargement. Gland puncture +. Spinal fluid 52.5 lymphocytes per cmm.; large mononuclears +. Treatment consisted of 4 doses of 4.0 gm. at 8, 17, and 7 day intervals or 400 mg. per kilo in 32 days. There were no untoward effects and during the period of observation patient was in excellent physical condition. The cervical lymph glands were reduced to a few shotty masses. A second examination of spinal fluid 5 weeks after the first showed 15 lymphocytes per cmm. and no large mononuclears were seen—a decrease of 70 per cent. A third examination, 66 days after admittance, showed a practically normal count of 3.1 lymphocytes per cmm., and no large mononuclears, and this value represented a decrease of 95 per cent from the original count. No further treatment is to be given this patient unless trypanosomes are found in the lymph glands or blood or the cell content of the spinal fluid increases.

A comparison of the amount of drug administered to the last 3 patients and the effect upon the spinal fluid obtained in each shows that in the case of Bolendo #9 10.0 mg. given in 5 doses of 2.0 gm. over a period of 34 days accomplished the same numerical result as 16.0 gm. in 4 doses of 4.0 gm. in 29 and 32 days given to N'Kusu #44 and N'Siola #32—that is, decreases of 68 and 70 per cent in the cell counts. However, it should be noted that the original examination of the spinal fluids of N'Kusu #44 and N'Siola #32 showed a more serious cerebrospinal involvement as indicated by the presence of large mononuclears. In addition, the effect of the treatment given to N'Siola #32 extended over a longer period than that of the same amount given to N'Kusu #44; the latter patient, however, presumably had a more difficult infection to influence as he came from a "virulent" district.

Fulu # 29.—Small, undernourished, debilitated, and anemic boy. Mental condition normal; tongue tremor. Scabies; pustular skin infection; filariasis. 25.5 kilos. Marked general glandular enlargement. Gland puncture ++. Spinal fluid 142.5 lymphocytes per cmm.; large mononuclears +. He was given 8 doses of 1.0 gm. at intervals of 3 to 9 days over a period of 33 days—so that he received a total amount of 8.0 gm. or 314 mg. per kilo. The second lumbar puncture, 5 weeks after the beginning of treatment, showed a cell count of 22.5 lymphocytes per cmm.—a decrease of 84 per cent and no large mononuclears were seen. A second course of treatment of 1.0 gm. per week for 7 injections was begun a month later. During the period of observation, the tongue tremor disappeared, and there

was a marked reduction in size and induration of the lymph glands with a moderate improvement in general physical condition.

Koloshi # 35.—Laborer. Very weak and anemic; unable to work. Dull mental state. Slight edema of upper eyelids and suggestive fullness of lower jaw. Marked tongue tremor; slight tremor of extended hands; greatly exaggerated knee jerks. Ankylostomiasis; chronic amœbiosis. 44.5 kilos. Slight general glandular enlargement. Gland puncture +. Spinal fluid 285 lymphocytes per cmm.; large mononuclears +; mulberry cells +. He was given 4 doses of 5.0 gm. at intervals of 7, 15, and 7 days, or 20.0 gm. in 29 days (449 mg. per kilo). No untoward effects were observed after individual doses or following the course of treatment. 5 weeks after the beginning of treatment, a second spinal fluid examination showed a cell count of 12.5 lymphocytes per cmm., a decrease of 96 per cent, and in addition only 1 large mononuclear cell was seen. Clinically, there was also a marked improvement; the tremors had disappeared, the knee jerks were practically normal, the edema of the eylids and lower jaw had disappeared, his mental condition appeared normal, and he was much stronger. The change was so striking that it was decided to defer further treatment until a third examination of the spinal fluid could be made. This was accomplished a month later and showed a cell count of 8 lymphocytes per cmm. with 1 large mononuclear seen in the counting chamber. Apparently the disease had not advanced during this time and, therefore, it was decided to omit further treatment until indicated by lymph gland, blood, or spinal fluid findings.

These patients represent typical cases of various degrees of advanced trypanosomiasis. The outstanding features of the treatment of the group are the marked and rapid diminution of the cells of the spinal fluid after 3 or 4 medium sized doses of tryparsamide and a conspicuous physical and mental improvement. Amounts of from 150 to 449 mg. per kilo of body weight given in an average of 4 weeks caused a prompt decrease in the cell content of from 68 to 96 per cent in 3 patients representing slight, moderate, and marked cerebrospinal involvement who showed continued improvement with no further treatment as indicated by lumbar punctures 12 and 9 weeks after admittance. In one patient, a third count showed a slight increase in cells above the second so that treatment was resumed. The 3 remaining patients of the group were given further treatment as the spinal fluids were slightly abnormal after the first course of tryparsamide and there was no means of ascertaining the duration of the therapeutic effect.

Previously Untreated Advanced Cases.

2 Courses of Treatment.

The effect of repeated doses of tryparsamide upon the spinal fluid of a second group of previously untreated patients is shown in Table XXIV. The cell content of the spinal fluid varied from 15 to 450 lymphocytes per cmm. and 6 specimens contained large mononuclears and mulberry cells. The order of arrangement of cases in the accompanying table corresponds to the initial cell count. In general, those patients with less marked abnormality of the spinal fluid showed a secondary anemia, some loss of weight, weakness, an increased pulse rate, and fever with slight nervous symptoms, while in the more advanced cases these symptoms were markedly exaggerated with the addition in certain individuals of pronounced mental disturbances. Trypanosomes were found in the cervical lymph glands of each patient.

Two general systems of treatment were followed in this group—the administration of 2 to 5 doses of 2.0 to 4.0 gm. at weekly intervals or semi-weekly injections of 2.0 gm. A second course of treatment was given approximately a month later. The first dose of the drug produced a sterilization of lymph glands and peripheral blood as previously described. The following abstracts include the important features of individual cases.

Effect upon the Spinal Fluid.—The first 3 patients in Table XXIV showed a slight increase in the cells of the spinal fluids, while the second 3 showed more of an increase and in addition came from a "virulent" district. The last 5 patients are examples of very advanced cases of trypanosomiasis as the cell counts of the spinal fluid indicate.

Bosc # 22.—Adult woman complaining of indefinite pains and general malaise. Fair state of nutrition; markedly anemic; exaggerated deep reflexes; mental condition normal. Marked cervical adenitis. Gland puncture +. Spinal fluid 15 lymphocytes per cmm. 56 kilos. She was given 6.0 gm. of tryparsamide or 107 mg. per kilo in 2 doses of 3.0 gm. a week apart. 33 days after the first dose, a second lumbar puncture was done; as the spinal fluid was considerably contaminated with blood, the only comment upon the approximate count of 22 lymphocytes per cmm., is that the pathological condition of the spinal fluid had not materially altered. Physically, there was considerable improvement during this

TABLE XXIV.

Cell Changes of Spinal Fluid in Advanced Cases Treated with Repeated Doses of Tryparsamide.
(No Previous Treatment.)

Case No.	Name.	First course of tryparsamide. Total.	Spinal fluid before treatment.	Spinal fluid after treatment. Per cent change.	2nd course tryparsamide.	3rd L. P. Per cent change.
22	Bose..... 56 kg.	2 x 3.0 gm. 6.0 gm. in 7 days. 107 mg per kg.	15	22(?) (33 days) R.B.C.	4 x 5.0 gm. 20.0 gm. in 19 days. 357 mg. per kg.	6.2 (70 days) 59% ‡
26	M'Pala .... 62 kg.	3 x 3.0 gm. 9.0 gm. in 14 days. 145 mg. per kg.	15	5.6 (30 days) 63%	4 x 5.0 gm. 20.0 gm. in 27 days. 323 mg. per kg.	4.0 (71 days) 73% ‡
6	Nembezi.... 46 kg.	5 x 2.0 gm. 10.0 gm. in 27 days. 220 mg. per kg.	(?)R.B.C.	20 (41 days)	4 x 4.0 gm. 16.0 gm. in 21 days. 348 mg. per kg.	2.0 (98 days) 90% ‡
14	Tono..... 45 kg.	4 x 2.0 gm. 8.7 gm. 1 x 0.7 " 193 mg. per kg. in 28 days.	20.2	7.5 (35 days) 1 l.m. 62%	3 x 4.0 gm. 12.0 gm. in 14 days. 267 mg. per kg.	9.4 (79 days) 53% §
16	Masimango.. 48 kg.	5 x 2.0 gm. 10.0 gm. in 28 days. 208 mg. per kg.	35	7(?) (37 days)† 80%	3 x 4.0 gm. 12.0 gm. in 14 days. 250 mg. per kg.	9.4 (79 days) 4 l.m. 73% ‡
15	Babuli..... 57.5 kg.	5 x 2.0 gm. 10.0 gm. in 28 days. 174 mg. per kg.	70 l.m+	30 (37 days) 2 l.m. 57%	4 x 4.0 gm. 16.0 gm. in 21 days. 278 mg. per kg.	20 (81 days) 1 l.m 71% ‡

24	Miaku*..... 46.5 kg.	2 x 4.0 gm. in 7 days.	8.0 gm. 172 mg. per kg.	305 l.m.++, m.++	25 (35 days) 2 l.m. 92%	5 x 2.0 gm. 10.0 gm. in 29 days. 239 mg. per kg.	16 (80 days) 4 l.m. 95% ‡
25	Madina..... 48 kg.	3 x 4.0 gm. in 14 days.	12.0 gm. 250 mg. per kg.	300 l.m.++, m.++	57.5 (37 days) 81%	8 x 2.0 gm. 16.0 gm. in 24 days. 333 mg. per kg.	14 (74 days) 4 l.m. 95% ‡
20	Kiale*..... 47 kg.	4 x 4.0 gm. in 21 days.	16.0 gm. 340 mg. per kg.	387.5 l.m.+, 2m.	35 (32 days) 91%	8 x 2.0 gm. 16.0 gm. in 24 days. 340 mg. per kg.	33 (78 days) 3–4 l.m. 9 % ‡
17	Bwaba..... 51.5 kg.	4 x 3.0 gm. in 21 days.	12.0 gm. 233 mg. per kg.	450 l.m.++, m.++		1 x 2.0 gm. 2.0 gm. 38 mg. per kg.	22.5 (60 days) 2 l.m. 95% §
10	Makengo.... 41.5 kg.	1 x 1.0 gm. 1 x 2.0 “ 1 x 3.0 “ 1 x 4.0 “ 1 x 5.0 “ in 24 days.	15.0 gm. 361 mg. per kg.	247.5 l.m.++, m.++	70 (38 days) 71%	1 x 2.0 gm. 1 x 0.5 “ 5 x 1.0 “ } 7.5 gm. in 30 days. 180 mg. per kg.	20.4 (86 days) 3 l.m. 92% ‡

* Previous treatment suspected.
† Trace of microscopic blood.
‡ Marked clinical improvement.
§ Moderate clinical improvement.

period and the reflexes had become normal. A second course of approximately 3 times the amount of the first was begun after an intermission of 4 weeks; 4 doses of 5.0 gm. or 357 mg. per kilo were given in 19 days. Her general condition continued to be good and she left the hospital. The cervical lymph glands were reduced to small, indurated masses. The third lumbar puncture 10 weeks after the beginning of treatment showed 6.2 lymphocytes per cmm. in the spinal fluid—a decrease of 59 per cent from the original cell count.

M'Pala ♯ 26.—Wife of a laborer. Good general physical and mental condition; anemic; malaria; general glandular enlargement. Gland puncture +. Spinal fluid 15 lymphocytes per cmm. 62 kilos. She was given 3 weekly doses of 3.0 gm., or 145 mg. per kilo in 14 days. The second examination of the spinal fluid, 30 days after the beginning of treatment, showed a cell content of 5.6 lymphocytes per cmm., or a decrease of 63 per cent. The second course of tryparsamide consisted of 4 weekly doses of 5.0 gm. or 323 mg. per kilo in 27 days—double the amount of the first course and given in twice the time. The succeeding spinal fluid examination 10 weeks after admittance was practically normal, 4 lymphocytes per cmm. During the period of observation, she was in excellent physical condition except for a short malarial attack; there was a marked regression of the adenitis.

Nembezi ♯ 6.—Wife of a soldier. Complained of deep pains in forearms and legs and of feeling sick for 2 months. Well nourished but markedly anemic; normal mental status. Slight cervical adenitis. Gland puncture +. Spinal fluid contaminated with blood. 46 kilos. Treatment was begun with 5 weekly doses of 2.0 gm. or 220 mg. per kilo in 27 days; the subjective symptoms rapidly disappeared and there was a marked improvement in the blood count. A lumbar puncture 6 weeks after admittance showed a cell count of 20 lymphocytes per cmm. A second course of tryparsamide of 4 weekly doses of 4.0 gm. was started after an intermission of 5 weeks; 16.0 gm. or 348 mg. per kilo was given in 21 days. The third lumbar puncture, 14 weeks after admittance, revealed a normal cell count—2 lymphocytes per cmm. She continued in excellent physical condition; the cervical lymph nodes became small and indurated.

Tono ♯ 14.—Boy from a "virulent" region. Fair state of nutrition but extremely weak and anemic. Discrete papulo-vesicular eruption over arms, wrists, backs of hands, and thighs. Normal mental status. Marked cervical adenitis. Gland puncture ++. Spinal fluid 20.2 lymphocytes per cmm. 45 kilos. He was given 5 weekly doses of 2.0 gm. or 193 mg. per kilo in 28 days (on injection incomplete). The eruption cleared during the first 2 weeks and there was a well marked general physical improvement; the cervical lymph glands became small and indurated. The second spinal fluid examination made 5 weeks after admittance showed 7.5 lymphocytes per cmm. (1 large mononuclear)—a decrease of 62 per cent. After an intermission of 2 weeks, treatment was resumed with 3 weekly doses of 4.0 gm. or 267 mg. per kilo, a slightly larger amount than the first course and administered in 2 weeks as contrasted with 4. The third lumbar puncture 11 weeks after admittance and approximately 3 weeks after the last dose of tryparsamide showed practically no change in the spinal fluid—9.4 lymphocytes per

cmm. No trypanosomes were found in the centrifuged specimens of the spinal fluid. Tryparsamide treatment was resumed

Masimango # 16.—Laborer from a "virulent" region. Fair state of nutrition but anemic and weak; unable to work. Dulled mental condition; tremor of tongue and extended hands; marked exaggeration of deep reflexes. Cervical glands enlarged and firm. Gland puncture ++. Spinal fluid 35 lymphocytes per cmm. 48 kilos. He was given 5 weekly doses of 2.0 gm. or 208 mg. per kilo in 28 days. There was a prompt physical and mental improvement with disappearance of tremors and exaggerated reflexes during the period of treatment. A second lumbar puncture 5 weeks after admittance showed 7 lymphocytes per cmm., a decrease of 80 per cent. After an interval of 2 weeks, a second course was begun in which 3 doses of 4.0 gm. or 250 mg. per kilo were given in 14 days. A third lumbar puncture was done 11 weeks after admittance and approximately 3 weeks after the last dose of tryparsamide; the spinal fluid contained 9.4 lymphocytes per cmm. and 4 large mononuclears were seen; no trypanosomes were found in centrifuged specimens. Although the third spinal fluid examination showed no improvement beyond that observed in the second, the clinical change was well marked, and the patient returned to work during the second course of treatment. Tryparsamide treatment was resumed.

Babuli # 15.—Laborer from a "virulent" district. Weak and anemic but not emaciated; unable to work. Irritable mental state; leg abscess and malaria; enlarged soft cervical glands. Gland puncture ++. Spinal fluid 70 lymphocytes per cmm.; large mononuclears +. 57.5 kilos. He was given 5 weekly doses of 2.0 gm. or 174 mg. per kilo in 28 days. In spite of a large leg abscess which developed during this period, there was a pronounced physical and mental improvement and a lumbar puncture 5 weeks after admittance showed a cell count of 30 lymphocytes per cmm.—a decrease of 57 per cent and in addition, only 2 large mononuclears were seen. No trypanosomes were found in centrifuged specimens of spinal fluid. The cervical adenitis had disappeared leaving a few tiny indurated glands. Treatment was resumed in 2 weeks with a second course of 4 weekly doses of 4.0 gm. or 278 mg. per kilo in 21 days, that is, 6.0 gm. more than his first course. His spinal fluid examined 12 weeks after admittance showed a further diminution of lymphocytes from 30 to 20 per cmm. or a decrease of 71 per cent from the original count of 70. In this last examination, moreover, only 1 large mononuclear was seen as compared with several in the first and 2 in the second examination. The clinical improvement was well marked and patient was able to return to work. Tryparsamide treatment was resumed because of the continued abnormal cell content of the spinal fluid.

Miaku # 24.—Soldier on sick leave. Complained of headache, indefinite pains in arms and legs, general malaise. Thin but not emaciated; very weak and anemic; dull mental status; beginning lethargy (?), marked tongue tremor, and inconstant tremor of extended hand; greatly exaggerated knee jerks; marked general glandular enlargement. Gland puncture +++. Spinal fluid 305 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. 46.5 kilos. He was given 2 doses of

4.0 gm. or 172 mg. per kilo of body weight in 7 days. Treatment was then stopped because of the development of visual disturbances; the nervous and mental symptoms, however, had materially improved and he felt much better physically. A second lumbar puncture 5 weeks after admittance showed a remarkable drop in the cells of the spinal fluid, from 305 to 25 or 92 per cent, and in addition no mulberry cells and only 2 large mononuclears were seen. Following an intermission of 6 weeks, treatment was resumed during a febrile attack which had lasted 2 weeks (trypanosomiasis?). Weekly doses of 2.0 gm. were given with beneficial results, for after 2 doses, there was a definite general improvement with cessation of fever. After 5 doses had been given in 29 days, a third lumbar puncture showed a further diminution of cells—16 lymphocytes per cmm. with 4 large mononuclears seen.

Madina № 25.—Laborer. Well nourished but very anemic; mentally affected with beginning lethargy; pronounced tremors of tongue and extended hands; malaria; marked cervical adenitis. Gland puncture ++. Spinal fluid 300 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. 48 kilos. He was given 3 doses of 4.0 gm. in 14 days or 250 mg. per kilo, which caused a decrease from 300 to 57.5 lymphocytes or 81 per cent. In addition, there was a gradual disappearance of the tremors present on admission and a pronounced mental and physical improvement. The cervical lymph glands were reduced to a few tiny indurated nodules. A second course of smaller doses was begun after an intermission of 4 weeks, consisting of 8 semi-weekly doses of 2.0 gm. or 333 mg. per kilo in 24 days. The third lumbar puncture 74 days after admittance showed the further marked diminution of cells to 14 lymphocytes per cmm., a drop of 95 per cent from the original count; 4 large mononuclears, however, were seen; no trypanosomes were found in centrifuged specimens. The clinical improvement beginning with the first course of tryparsamide was particularly marked and he returned to work, although in view of the residual abnormality of his spinal fluid further treatment was recommended.

Kiale № 20.—House servant. Complained of generalized pains and feeling very ill; not emaciated but weak and asthenic. Extremely dull mental state; marked tremor of tongue and of hands at rest; deep reflexes greatly exaggerated; tinea versicolor. Pronounced cervical adenitis. Gland puncture +. Spinal fluid 387.5 lymphocytes per cmm.; large mononuclears +; 2 mulberry cells. 47 kilos. He was given 4 weekly doses of 4.0 gm. or 340 mg. per kilo in 21 days. The second examination of his spinal fluid made 5 weeks after the first showed a decrease from 387.5 to 35 lymphocytes per cmm. or 91 per cent. At this time his mental condition appeared normal, there were no tremors and the knee jerks were but slightly exaggerated; there was also a well marked physical improvement. A second course of treatment, begun 25 days after the first, consisted of 2 doses of 2.0 gm. per week for 8 injections so that he received the same amount of drug, 16.0 gm., in practically the same time as the first course. The third lumbar puncture 11 weeks after admittance showed no further change in the number of lymphocytes, 33 per cmm.; but 3 or 4 large mononuclear cells were seen and further treatment was recommended. He returned to work and seemed mentally and physically able to do so.

Bwaba # 17.—Laborer from a "virulent" region. Poor state of nutrition, very weak and anemic. Discrete papulo-vesicular eruption over extensor surfaces of forearms. Mental deterioration but no lethargy; marked tremor of tongue and hands at rest; greatly exaggerated knee jerks; slightly enlarged firm cervical glands. Gland puncture +. Spinal fluid 450 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. 51.5 kilos. He was given 4 weekly doses of 3.0 gm. or 233 mg. per kilo in 21 days with a marked initial clinical improvement. The tremors disappeared, his knee jerks became normal, and the eruption cleared rapidly. During the week following the last dose, however, visual disturbances developed and ultimately there occurred a brawny, indurated swelling of the lower left leg of an uncertain nature and a considerable loss of weight; lumbar puncture was deferred. A second course of smaller doses was begun 5 weeks later; 2 days after the first injection of 2.0 gm., a lumbar puncture was done. It was found that the spinal fluid contained only 22.5 lymphocytes per cmm., a decrease of 95 per cent; 2 large mononuclears were seen. Treatment was continued for 5 additional weekly injections with no further ill effects. There was definite improvement of the mental and nervous symptoms of this patient but less physical benefit than in the other patients of the group.

Makengo # 10.—Young adult laborer. Thin, anemic, and weak; slight edema upper eyelids; discrete papular eruption over forearms, thighs, and legs; uncertain confused mental state; slight lethargy. Tremor of tongue; exaggerated deep reflexes. Cervical adenitis; gland puncture +++. Spinal fluid 247.5 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. 41.5 kilos. He was given 5 doses of tryparsamide increasing in size from 1.0 to 5.0 gm. so that he received 15.0 gm. or 361 mg. per kilo in 24 days. The second dose of 2.0 gm. was given 3 days after the first, while the succeeding doses of 3.0, 4.0, and 5.0 gm. were given at weekly intervals. There were no untoward effects until after the last dose when visual disturbances appeared and treatment was stopped. A marked clinical improvement, however, had occurred, with disappearance of lethargic symptoms, edema of eyelids, cutaneous eruption, and tongue tremor, and there was a considerable increase in weight. 38 days after admittance, the spinal fluid contained 70 lymphocytes per cmm., a decrease of 71 per cent with no large mononuclears and mulberry cells. A second course of smaller doses of tryparsamide was begun after an interval of 5 weeks, consisting of one 2.0, one 0.5, and five 1.0 gm. doses given on an average of every 5 days so that the patient received 7.5 gm. or 180 mg. per kilo in 30 days. A third lumbar puncture, 12 weeks after admittance, showed a further marked diminution of cells—20.4 lymphocytes per cmm., or a decrease of 92 per cent from the original cell count (3 large mononuclears were seen in the last examination). The physical improvement of this patient was pronounced as indicated by a gain in weight of 7.5 kilos, while the tongue tremor and mental symptoms present on admittance were alleviated.

Considering this group as a whole, the outstanding feature of the treatment is the extremely rapid and marked diminution in the number

of cells of the spinal fluid together with a physical and mental improvement after short courses of tryparsamide. Decreases of from 57 to 92 per cent in the cell counts occurred in from 4 to 5 weeks after the beginning of treatment which in most cases consisted of 2, 3, or 4 doses of 3.0 to 4.0 gm. given at weekly intervals. Second courses of treatment given to 10 patients were followed by further improvement of the spinal fluid in 7 cases while in 3 the diminished cell count remained approximately unchanged. Associated with the changes of the cell content of the spinal fluid was a pronounced beneficial effect upon the physical condition and a similar improvement of nervous and mental symptoms.

Previously Treated Advanced Cases.

This group consisted of 10 patients, 7 of whom were being treated with atoxyl and tartar emetic and 3 the nature of whose previous treatment was unknown. The cell counts of the spinal fluid ranged from 32 to 572 lymphocytes per cmm. and in 6 instances large mononuclears and mulberry cells were seen. The majority of the patients showed pronounced clinical manifestations of an advanced condition. Trypanosomes were found in the cervical lymph glands of the 3 patients who had been treated at some previous time, so they may be considered both as relapsed and non-cured cases; the usual preliminary sterilizing action of tryparsamide was shown by negative examinations of the lymph gland juice of these patients. No one system of treatment was followed in this group but in general doses of various sizes were given at weekly or semi-weekly intervals.

Effect upon the Spinal Fluid.—The effect of treatment with tryparsamide will be considered first in regard to the changes produced in the spinal fluid as shown in Table XXV.

Yali № 13 had received 2.0 gm. soamin and 0.4 gm. tartar emetic during the 4 weeks previous to his admittance. He was weak, anemic, and unable to work. Dull mental condition; marked coarse tremor of the tongue and extended hands; exaggerated deep reflexes. Spinal fluid contained 32 lymphocytes per cmm. 51.5 kilos. He was treated at weekly intervals with 2 doses of 3.0 gm. and 2 doses of 4.0 gm. of tryparsamide, a total of 14.0 gm. or 272 mg. per kilo in 21 days. Clincal improvement was extremely striking by the third week; he was appreciably stronger and mentally seemed normal; there were no tremors and the reflexes

were normal. A second lumbar puncture, 32 days after the first showed 4.6 lymphocytes per cmm. or a decrease of 86 per cent. He remained in excellent condition over an observation period of 3 months during which time examinations of gland juice and centrifuged blood were negative. A third examination of the spinal fluid 74 days after admittance showed the practically normal cell count of 3.4 lymphocytes per cmm. He was able to return to work.

Eali № 1 had received 1 dose of 0.75 gm. of atoxyl 4 days previously. On that day, his spinal fluid contained 80 lymphocytes per cmm. He was thin, weak, and anemic; general glandular enlargement and slightly exaggerated deep reflexes. Mental condition normal. 57 kilos. He was given 2 doses of 2.0 gm. of tryparsamide with a two weeks' interval, or 70 mg. per kilo. Spinal fluid examination 4 weeks after beginning treatment showed a cell content of 62.5 lymphocytes per cmm.—a decrease of 22 per cent, thus indicating that small amounts of tryparsamide, may, in certain cases, have a slight but definite effect upon the spinal fluid and that this effect may endure for an appreciable time. The patient's physical condition had definitely improved; there was a marked reduction of the cervical adenitis and the red cells and hemoglobin of the blood had increased considerably. Treatment was resumed at weekly intervals with 1 dose of 2.0 gm. and 2 doses of 4.0 gm., a total of 10 gm. or 175 mg. per kilo in 14 days. 2 days after the last dose, patient complained of visual dimness and treatment was stopped. During the next 3 weeks, he became quite ill; the condition was diagnosed clinically as an exacerbation of trypanosomiasis and death occurred 5 weeks after the cessation of treatment.

Bibaba № 2 had been treated with 2.5 gm. soamin and 0.4 gm. tartar emetic for 43 days but still showed marked weakness, anemia, general glandular enlargement, greatly exaggerated deep reflexes, and mental apathy. 59 kilos. Spinal fluid contained 37.5 lymphocytes per cmm. He was given 4 doses of 3.0-gm. at 2, 4, and 3 day intervals, a total of 12.0 gm. or 203 mg. per kilo in 9 days. 2 days after the last dose, patient complained of visual impairment and treatment was stopped. A second lumbar puncture done 4 weeks after the beginning of treatment showed 20 lymphocytes per cmm.—a decrease of 47 per cent. A month later, the patient became ill (trypanosomiasis relapse?) and 2 weekly doses of 2.0 gm. were given. There was no increase of residual visual symptoms but the general condition became worse and death occurred.

M'Boyo № 3 had received 2.0 gm. soamin and 0.2 gm. tartar emetic during 26 days preceding tryparsamide treatment. She was undernourished, anemic, and weak; there was a coarse tremor of the hands and greatly exaggerated deep reflexes; she showed mental deterioration and apathy and frequent periods of lethargy; cervical lymph glands barely palpable. 49 kilos. Spinal fluid contained 92.5 lymphocytes per cmm. with many large mononuclears (+++). She was given 4 doses of 3.0 gm. at 2, 4, and 3 day intervals—a total of 12.0 gm. or 245 mg. per kilo in 9 days. Visual disturbance began 2 to 3 days after the last dose and the drug was stopped. The spinal fluid examined 4 weeks after beginning treatment was found to contain only 7.5 lymphocytes per cmm.—a decrease of

TABLE XXV.

Cell Changes of the Spinal Fluid of Previously Treated Advanced Cases, Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	First course of tryparsamide. Total.	Spinal fluid before treatment.	Spinal fluid after treatment. Per cent change.	2nd course of tryparsamide. Total.	3rd L. P. Per cent change.
13	Yali*..... 51.5 kg.	2 x 3.0 gm. 14.0 gm. 2 x 4.0 " 272 mg. per kg. in 21 days.	32	4.6 (32 days) 86%		3.4 (74 days) 89%‖
1	Eali*..... 57.0 kg.	2 x 2.0 gm. 4.0 gm. in 13 days. 70 mg. per kg.	80	62.5 (27 days) 22%	1 x 2.0 gm. 10 gm. 2 x 4.0 " 175 mg. per kg. in 14 days.	†
2	Bibaba*..... 59.0 kg.	4 x 3.0 gm. 12.0 gm. in 9 days. 203 mg. per kg.	37.5	20 (27 days) 47% 1 m.	2 x 2.0 gm. 4.0 gm. in 8 days. 67 mg. per kg.	†
3	M'Boyo*.... 49.0 kg.	4 x 3.0 gm. 12.0 gm. in 9 days. 245 mg. per kg.	92.5 l.m.+++	7.5 (27 days) 92%	3 x 4.0 gm. 12.0 gm. in 14 days. 245 mg. per kg.	3.1 (90 days) 97%‖
4	Etumbu*.... 48.5 kg.	3 x 2.0 gm. 9.0 gm. 1 x 3.0 " 185 mg. per kg. in 9 days.	142.5 l.m.+	17.5 (27 days) 88%		‡
8	Tokamba*.. 39.0 kg.	1 x 4.0 gm. 20.0 gm. 8 x 2.0 " 513 mg. per kg. in 28 days.	100 l.m.++	7.5 (39 days) 93% 1 l.m.	6 x 2.0 gm. 12.0 gm. in 36 days. 286 mg. per kg.	§

7	Engondo*... 45.0 kg.	4 x 4.0 gm. in 32 days. 16.0 gm. 356 mg. per kg.	572 l.m.+++ m.+		4 x 2.0 gm. in 21 days. 8.0 gm. 178 mg. per kg.		25 (86 days) 3 l.m. 96%§
21	Monololo... 60.0 kg.	4 x 4.0 gm. in 21 days. 16.0 gm. 267 mg. per kg.	37.5	17.5 (32 days) 3 l.m. 53%	2 x 6.0 gm. 1 x 4.0 " in 29 days.	16.0 gm. 267 mg. per kg.	33 (77 days) l.m+ 12%††
27	K. Lukas... 48.0 kg.	3 x 5.0 gm. in 14 days. 15.0 gm. 313 mg. per kg.	327.5 l.m.+++ m.++	15 (31 days) 95%	8 x 2.0 gm. in 26 days.	16.0 gm. 333 mg. per kg.	41.5 (79 days) 8–9 l.m. 87%§
34	Langomo... 62.5 kg.	3 x 7.0 gm. in 21 days. 21.0 gm. 336 mg. per kg.	467.5 l.m.+++ m.+++	10 (35 days) 98%			**

* Under atoxyl and tartar emetic treatment when transferred to tryparsamide therapy.
† Died. Trypanosomiasis (?).
‡ Died. Amœbic dysentery—trypanosomiasis (?).
§ Improvement.
‖ Marked improvement.
** Marked improvement. Deserted.
†† Stationary state.

92 per cent and in addition no large mononuclear or mulberry cells were seen. By this time the visual disturbance had completely disappeared and there was a noticeable mental and physical improvement. She no longer showed any tendency toward a lethargic state and the tremors had disappeared although the deep reflexes continued to be exaggerated. Treatment was resumed after 3 weeks and 3 doses of 4.0 gm. were given at weekly intervals, so that the same amount of drug, 12.0 gm. or 245 mg. per kilo was given again but during a period of 14 instead of 9 days. No visual or other disturbances occurred and there was a continued striking clinical improvement. She appeared cheerful and happy, was able to take care of herself and answered questions intelligently, all of which contrasted sharply with her condition when first seen. A third examination of the spinal fluid 3 months after admittance contained 3.1 lymphocytes per cmm.—a practically normal count.

Etumbu ♀ *4* had been treated with 1.5 gm. soamin and 0.2 gm. tartar emetic during a period of 22 days. He was somewhat emaciated and very anemic. Mentally apathetic. 48.5 kilos. Spinal fluid contained 142.5 lymphocytes per cmm.; large mononuclears +. He was given 3 doses of 2.0 gm. and 1 dose of 3.0 gm. at 2, 4, and 3 day intervals—a total of 9.0 gm. or 185 mg. per kilo in 9 days. Visual impairment developed 3 to 4 days after the last dose and no further treatment was given. This effect was transitory and his sight became normal within 2 weeks. A second spinal fluid examination made 4 weeks after the first showed 17.5 lymphocytes per cmm. or a decrease of 88 per cent. At this time, however, amœbic dysentery developed and although emetine treatment resulted in negative stool examinations, he died 3 weeks later. The question of a trypanosomiasis exacerbation must also be considered as a possible cause of death.

Tokamba ♀ *8* was an old advanced case, who had been in the hospital for 9 months, during which time he had received 27 injections of 0.5 gm. soamin (13.5 gm.), and 29 injections of 0.1 gm. tartar emetic (2.7 gm.). His general state had not been improved. There was marked emaciation and weakness; anemia; greatly exaggerated deep reflexes; tremors of the tongue, hands, and feet; a typical shuffling gait and marked mental deterioration and apathy. 39 kilos. Spinal fluid contained 100 lymphocytes per cmm.; large mononuclears ++. He was given one dose of 4.0 gm. tryparsamide, followed by 8 doses of 2.0 gm. at 3 and 4 day intervals so that a total of 20.0 gm. or 513 mg. per kilo was administered in 28 days. No untoward effects were observed. A second spinal fluid examination, 39 days after the beginning of treatment, showed a markedly diminished number of cells—7.5 lymphocytes per cmm., or a decrease of 93 per cent and only 1 large mononuclear was seen. At this time, patient's physical condition was distinctly improved; he had gained weight and seemed brighter mentally but his nervous symptoms were still marked. Treatment was resumed 5 weeks later and he was given 6 weekly doses of 2.0 gm. or 286 mg. per kilo. The clinical improvement in this patient was not as striking as in the case of M'Boyo ♀ 3 but the mental deterioration and nervous symptoms at the time he was transferred to tryparsamide treatment were of a more advanced and serious character as well as of longer duration. In addition, he had been previously treated for many months.

Engondo # 7 had been under more or less constant observation for 7 years. She had been originally treated with salvarsan-copper, followed by atoxyl and tartar emetic, after which she deserted. She was readmitted to the hospital 4½ years later in a markedly advanced stage of the disease. During 2½ years, she received 63 injections of 0.5 gm. of atoxyl (31.5 gm.), 53 injections of 0.5 gm. soamin (26.5 gm.), and 114 injections of 0.1 gm. tartar emetic (11.4 gm.). Examination showed an extreme mental deterioration, typical shuffling gait, marked tremors of tongue, hands, and feet, and greatly exaggerated reflexes. She was wholly irresponsible with hallucinations and violent hysterical attacks and in addition was in a lethargic state much of the time. Thin but otherwise fair general physical condition. 45 kilos. Spinal fluid contained 572 lymphocytes per cmm. with numerous large mononuclears and mulberry cells. 4 doses of 4.0 gm. tryparsamide were given at weekly intervals, a total of 16.0 gm. or 356 mg. per kilo, in 32 days. There were no untoward effects but little mental improvement was observed. A second course of 6 weekly doses of 2.0 gm. was begun a month later and after 4 of these had been given a lumbar puncture was done (12 weeks after the beginning of tryparsamide treatment). The spinal fluid showed the markedly diminished cell count of 25 lymphocytes per cmm., a decrease of 96 per cent, and only 3 large mononuclears were seen. The patient's mentol condition, however, was still very abnormal; her disposition was unchanged and she still showed periods of lethargy. Her gait, however, had materially improved, the deep reflexes were less exaggerated; there were no tremors and 3 months after the beginning of treatment, there was a gain in weight of 4 kilos. The cerebrospinal involvement of this patient was extreme and of long duration, persisting in spite of continued atoxyl and tartar emetic treatment, and she thus represents a type of case considered as hopeless of therapeutic influence. Under these circumstances, therefore, a marked diminution of the cells of the spinal fluid with a certain degree of clinical improvement may be considered as a definite effect of tryparsamide treatment.

Monololo # 21, a soldier on sick leave, gave a history of having been treated for several months about a year before admittance. He was a well developed, well nourished man, with normal reflexes and no mental or nervous symptoms. Cervical adenitis; gland puncture ++. 60 kilos. Spinal fluid contained 37.5 lymphocytes per cmm. He was given 4 weekly doses of tryparsamide of 4.0 gm. each, or 267 mg. per kilo in 21 days with no untoward effects. 32 days after the administration of the first dose, a seond examination of the spinal fluid showed a cell content of 17.5 lymphocytes per cmm.—a decrease of 53 per cent; 3 large mononuclear cells were seen. A second course of treatment was begun 2 weeks after the first because the decrease of the spinal fluid cells had not been as great as in most of the other cases. Because of his size and excellent physical condition, the dose was increased to 6.0 gm. and he was given 2 doses 10 days apart. 10 days after the second dose, he complained of slight visual dimness which had occurred 2 or 3 days before. He did not return for 3 weeks and was then given 4.0 gm. 12 days later, or 11 weeks after the administration of the first dose of tryparsamide, the spinal fluid conained 33 lymphocytes per cmm. with several large mononuclears, a similar finding to that seen on admittance.

The correct interpretation of the last spinal fluid examination must take into account the type and nature of the infection, but, at all events, it is possible that with a greater number of regularly spaced doses, a more favorable effect might have been obtained. With this in view, it was decided to continue the treatment with weekly doses of 3.0 gm. over a longer period of time.

K. Lukas # 27, a house servant, had been treated for a long time in various localities. He was unable to work and complained of pains in his legs. Well nourished but anemic with a general glandular enlargement, tongue tremor and greatly exaggerated deep reflexes. Mental state normal. Gland puncture ++. 48 kilos. Spinal fluid contained 327.5 lymphocytes per cmm.; large mononuclears +++; mulberry cells ++. He was given 15.0 gm. or 313 mg. per kilo in 3 weekly doses of 5.0 gm. with no untoward effects. 31 days after admittance, his spinal fluid contained 15 lymphocytes per cmm., a decrease of 95 per cent. A second course of tryparsamide, consisting of 2 doses of 2.0 gm. per week, was begun 4 weeks later and he received 8 doses, a total of 16.0 gm. in 26 days, practically the same amount of the drug as in the first course but in 26 instead of 14 days. The third spinal fluid examination made 11 weeks after admittance showed a cell content of 41.5 lymphocytes per cmm., a decrease of 87 per cent from the original count but an increase above the second, and in addition 8 to 9 large mononuclears were seen. The physical condition of this patient was excellent and therefore it was decided to begin a third course of larger doses of 4.0 gm. administered once a week for at least 5 doses. Although the second course of tryparsamide had not been sufficient to produce a continued diminution of the spinal fluid cells, he was able to return to his work.

Langomo # 34 was a "vagabond" appearing first in one district and then in another and had been treated for an unknown period of time. He was a large, strong, powerfully built man, with a slight general glandular enlargement and markedly exaggerated deep reflexes. He showed great mental deterioration and apathy but was not lethargic. Gland puncture +. Spinal fluid contained 467.5 lymphocytes per cmm.; large mononuclears +++; mulberry cells +++. 62.5 kilos. He was given 3 doses of 7.0 gm. of tryparsamide in 21 days at 13 and 8 day intervals, a total of 21.0 gm. or 336 mg. per kilo with no untoward effects of any kind. There was a very evident mental improvement, however, shown by his rational answers to questions and the disappearance of mental apathy. The second spinal fluid examination, 5 weeks after the first, showed a cell content of only 10 lymphocytes per cmm., a decrease of 98 per cent and in addition no large mononuclear or mulberry cells were seen. Unfortunately no further observations on this patient could be made, as he ran away.

The striking feature of the treatment of this group of advanced cases is the marked and rapid diminution of the cells in the spinal fluid induced by a few doses of tryparsamide. Decreases in the cell count of from 47 to 98 per cent occurred in 4 to 5 weeks with the

exception of one patient, Eali #1, who was given only 2 fortnightly doses, and here there was a decrease of 22 per cent.

The system of therapy employed in the majority of instances consisted of 3 or 4 injections of 2.0 to 7.0 gm. at various intervals ranging from 3 to 14 days. The administration of doses at weekly or 10 day intervals was well borne with two exceptions but more frequent dosage caused visual disturbances in 3 instances which for the most part were transitory. In the case of Yali #13, the decrease of the cells in the spinal fluid induced by one course of tryparsamide continued over the observation period of $7\frac{1}{2}$ weeks after cessation of treatment; regarding Langomo #34, only the early effect of one course of treatment was observable as he deserted. The second course of treatment given to M'Boyo #3 caused a further diminution in the cell count of the spinal fluid and it is probable that a similar condition existed in the case of Engondo #7. It was not possible to obtain additional lumbar punctures from the 3 patients who died, and with Tokamba #8, it was deferred until further treatment had been given. In 2 patients, Monololo #21 and K. Lukas #27, the diminished cell content of the spinal fluid was not further influenced by the second course of tryparsamide and in one instance this may be partly explained by the irregular spacing and insufficient number of doses. Further, in judging the results of the treatment of this group of patients, the type and stage of the disease and the fact that prolonged previous treatment had been given must also be taken into consideration.

The mental and physical improvement which paralleled the changes in the spinal fluid were likewise striking and it should be pointed out that they occurred in all patients with 3 exceptions in whom the treatment was stopped.

GENERAL OBSERVATIONS.

Effect upon the Blood.—The changes in the blood counts of advanced cases of trypanosomiasis treated with one or two courses of repeated doses of tryparsamide were studied in 24 patients as shown in Tables XXVI to XXVIII. The first 17 patients in these tables were previously untreated, while the last 7 had been treated before. The amount of tryparsamide given to these patients ranged from 70 to 680 mg. per kilo; in general this was given in weekly doses of 2.0 to

TABLE XXVI.

Changes in Blood Counts, Advanced Cases, Treated with Repeated Doses of Tryparsamide. (One Course.)
(No Previous Treatment.)

Case No.	Name.	Doses of tryparsamide.	Total.	Blood count before treatment.		Last blood count after treatment.	Change.	Interval between counts.
							per cent	*days*
18	Loyoko.....	3 x 3.0 gm.	9.0 gm.	R.B.C.	2,720,000	4,800,000	+ 76	82*
	60.0 kg.	in 28 days.	150 mg. per kg.	Hb.	45%	80%	+ 78	
				W.B.C.	7,500	8,000	+ 7	
49	Makalamba.....	9 x 1.0 gm.	9.0 gm.	R.B.C.	4,320,000	4,640,000	+ 7	47†
	52.5 kg.	in 29 days.	171 mg. per kg.	Hb.	70%	75%	+ 7	
				W.B.C.	11,000	10,000	− 9	
29	Fulu.....	8 x 1.0 gm.	8.0 gm.	R.B.C.	4,160,000	4,160,000	−	46‡
	25.5 kg.	in 33 days.	314 mg. per kg.	Hb.	55%	50%	− 9	
				W.B.C.	4,500	11,000	+144	
32	N'Siola.....	4 x 4.0 gm.	16.0 gm.	R.B.C.	4,160,000	5,120,000	+ 23	73
	40.0 kg.	in 32 days.	400 mg. per kg.	Hb.	60%	75%	+ 25	
				W.B.C.	8,500	8,000	− 6	
35	Koloshi.....	4 x 5.0 gm.	20.0 gm.	R.B.C.	3,840,000	5,120,000	+ 33	45§
	44.5 kg.	in 29 days.	449 mg. per kg.	Hb.	45%	60%	+ 34	
				W.B.C.	5,000	8,000	+ 60	

* Filariasis.
† Syphilis; malaria.
‡ Scabies; pustular skin infection; filariasis.
§ Chronic ankylostomiasis and amœbiosis.

5.0 gm. The second blood count was made from 3 to 14 weeks after the first. The red and white cell counts and hemoglobin determination before and after treatment are also tabulated separately for convenience of comparison (Tables XXIX, XXX, XXXI).

The striking feature of the initial blood counts was the marked degree of secondary anemia. 17 patients had red cell counts of below 4 million, 6 between 4 and 4½ million, and only 1 had a normal number of red cells. The hemoglobin was likewise diminished; 14 determinations were 50 per cent or lower, 8 were between 50 and 60 per cent, and 2 were 70 per cent. There was a leukopenia of below 6,000 white cells in 12 instances, in 3 between 6 and 8 thousand, while 9 patients had counts of between 8 and 12 thousand.

The effect of tryparsamide treatment upon the red cell count is seen in Table XXIX. In only one instance (Fulu #29) was an improvement not observed, while in 23 patients, the red cells were increased from 7 to 100 per cent. In 13 patients this increase amounted to more than 20 per cent and in 10 patients from 7 to 19 per cent.

The hemoglobin changes were even more striking (Table XXX). There was an increase of 7 to 78 per cent in 23 patients and a decrease of 9 per cent in one patient (Fulu #29). 11 patients showed an increase of 40 per cent or more, while in 6 others the increase was above 20 per cent.

The white cell counts were also influenced by the tryparsamide treatment as shown in Table XXXI. There were both increases and decreases but in general there was a definite tendency toward a normal count as may be seen by comparing the 4th and 5th columns in this table.

These results are in accord with the observations on the blood picture of patients in the earlier stages of the disease treated with single or repeated doses of tryparsamide and are indicative of a favorable response to the drug.

Effect upon the Weight.—The changes in weight of 28 advanced cases treated with repeated doses of tryparsamide are given in Tables XXXII and XXXIII. The 18 patients in Table XXXII are those who had had no previous treatment, while the 10 in Table XXXIII had been treated before for varying periods of time. The first 7 patients in Table XXXII were given 1 course of tryparsamide,

TABLE XXVII.

Changes in Blood Counts, Advanced Cases, Treated with Repeated Doses of Tryparsamide. (Two Courses.)
(No Previous Treatment.)

Case No.	Name.	Doses of tryparsamide. Total.	Blood count before treatment.	Last blood count after treatment.	Change.	Interval between counts.
					per cent	*days*
22	Bose.....	2 x 3.0 gm. 6.0 gm	R.B.C. 2,560,000	5,120,000	+100	85
	56.0 kg.	in 7 days.	Hb. 52 %	75 %	+ 44	
		4 x 5.0 gm. 20 "	W.B.C. 5,200	8,400	+ 61	
		in 19 days.				
		26.0 gm. 464 mg. per kg.				
26	M'Pala.....	3 x 3.0 gm. 9.0 gm.	R.B.C. 4,136,000	5,120,000	+ 24	71*
	62.0 kg	in 14 days.	Hb. 55 %	80 %	+ 45	
		4 x 5.0 gm. 20.0 "	W.B.C. 9,600	10,000	+ 4	
		in 27 days.				
		29.0 gm. 468 mg. per kg.				
6	Nembezi.....	5 x 2.0 gm. 10.0 gm.	R.B.C. 3,616,000	4,160,000	+ 15	93†
	46.0 kg.	in 27 days.	Hb. 40 %	65 %	+ 63	
		4 x 4.0 gm. 16.0 "	W.B.C. 8,500	6,600	− 22	
		in 21 days.				
		26.0 gm. 568 mg. per kg.				
14	Tono.....	4 x 2.0 gm. 8.7 gm.	R.B.C. 3,352,000	4,000,000	+ 19	81
	45.0 kg.	1 x 0.7 "	Hb. 48 %	75 %	+ 56	
		in 28 days.	W.B.C. 6,000	5,200	− 13	
		3 x 4.0 gm. 12.0 "				
		in 14 days.				
		20.7 gm. 460 mg. per kg.				

16	Masimango..... 48.0 kg.	5 x 2.0 gm. 10.0 gm. in 28 days. 3 x 4.0 gm. 12.0 " in 14 days. 22.0 gm. 458 mg. per kg.	R.B.C. Hb. W.B.C.	3,040,000 50 % 7,500	5,040,000 70 % 9,000	+ 66 + 40 + 20	84
15	Babuli..... 57.5 kg.	5 x 2.0 gm 10.0 gm. in 28 days. 4 x 4.0 gm. 16.0 " in 21 days. 26.0 gm. 452 mg. per kg.	R.B.C. Hb. W.B.C.	3,840,000 46 % 4,000	4,320,000 65 % 8,000	+ 13 + 41 +100	81‖
24	Miaku ..... 46.5 kg.	2 x 4.0 gm. 8.0 gm. in 7 days. 5 x 2.0 gm. 10.0 " in 29 days. 18.0 gm. 411 mg. per kg.	R.B.C. Hb. W.B.C.	3,104,000 52 % 5,400	3,200,000 65 % 4,400	+ 3 + 25 − 18	78§
25	Madina..... 48.0 kg.	3 x 4.0 gm. 12.0 gm. in 14 days. 8 x 2.0 gm. 16.0 " in 24 days. 28.0 gm. 583 mg. per kg.	R.B.C. Hb. W.B.C.	3,728,000 50 % 5,550	4,000,000 70% 7,800	+ 7 + 40 + 40	74*
20	Kiale..... 47.0 kg.	4 x 4.0 gm. 16.0 gm. in 21 days. 8 x 2.0 gm. 16.0 " in 24 days. 32.0 gm. 680 mg. per kg.	R.B.C. Hb. W.B.C.	4,000,000 60 % 12,000	4,320,000 70 % 9,000	+ 8 + 17 − 25	81‡

TABLE XXVII—*Continued.*

Case No.	Name.	Doses of tryparsamide. Total.	Blood count before treatment.	Last blood count after treatment.	Change.	Interval between counts.
					per cent	*days*
17	Bwaba..... 51.5 kg.	4 x 3.0 gm. 12.0 gm. in 21 days. 5 x 2.0 gm. 10.0 " in 28 days. 22.0 gm. 427 mg. per kg.	R.B.C. 2,400,000 Hb. 42 % W.B.C. 12,000	4,060,000 55% 8,000	+ 69 + 31 − 33	87§
10	Makengo..... 41.5 kg.	1 x 1.0 gm. 1 x 2.0 " 1 x 3.0 " 1 x 4.0 " 1 x 5.0 " } 15.0 gm. in 24 days. 1 x 2.0 gm. 1 x 0.5 " 5 x 1.0 " } 7.5 gm. in 30 days. 22.5 gm. 541 mg. per kg.	R.B.C. 2,940,000 Hb. 48 % W.B.C. 10,000	4,480,000 60 % 7,200	+ 52 + 25 − 28	88
9	Bolendo..... 49.0 kg.	5 x 2.0 gm. 10.0 gm. in 34 days. 3 x 4.0 gm. 12.0 " in 18 days. 22.0 gm. 449 mg. per kg.	R.B.C. 2,912,000 Hb. 55 % W.B.C. 6,200	4,000,000 65 % 7,000	+ 37 + 18 + 13	88

* Malaria.
† Malaria (?).
‡ Tinea versicolor.
§ Trypanosomiasis exacerbation (?).
‖ Malaria; leg abscess.

while the remainder, 21 (Tables XXXII and XXXIII), received 2 courses averaging 1 month each and separated in most instances by an interval of 4 to 6 weeks. The observation periods range from 4 to 16 weeks.

Taking up first the weight changes in the previously untreated patients, it is seen that 14 showed a gain of from 0.5 to 8.5 kilos during 8 to 14 weeks; 2 patients showed no change—Loyoko #18, a well nourished adult, and Fulu #29, a small boy with an extensive skin infection and filariasis; and 2 others lost weight—Babula #15, who had a large leg abscess and an attack of estivo-autumnal malaria, and Bwaba #17, who probably had an exacerbation of the disease together with visual disturbances. The smaller increases of 0.5 to 1.0 kilo occurred in 7 patients, 2 of whom had malaria, 1 visual disturbances and probably an exacerbation of trypanosomiasis, and 1 chronic amœbic dysentery and ankylostomiasis, while 7 patients showed a gain of from 3.0 to 8.5 kilos.

The 10 patients in Table XXXIII were previously treated with other drugs as has already been described. 3 of them became very ill during the period of observation, lost weight, and eventually died (Eali #1, Bibaba #2, and Etumbu #4). The other 7 patients showed increases of weight of from 3.0 to 7.0 kilos except Langomo #34, who was in a good state of nutrition on admittance and gained only 0.5 kilo during 6 weeks.

The general beneficial effect of tryparsamide in advanced cases of trypanosomiasis, as indicated by increases of weight in the majority of patients, is similar to that observed after the administration of single and repeated doses in early stages of the disease. However, since the advanced cases were usually more emaciated, the improvement is relatively more pronounced.

Untoward Effects.—The administration of single or repeated doses of tryparsamide in all types of human trypanosomiasis represented by the 77 cases treated in Léopoldville was singularly free from immediate or delayed so called toxic symptoms or reactions, such as nausea, fever, headaches, diarrhœa, renal disturbances, jaundice, petechial hemorrhages, cutaneous reactions of various types, and syncope which may occur after the administration of various arsenical agents. A continued clinical improvement was characteristic of the tryparsa-

TABLE XXVIII.

Changes in Blood Counts, Advanced Cases, Treated with Repeated Doses of Tryparsamide.
(Previous Treatment.)

Case No.	Name.	Doses of tryparsamide. Total.	Blood count before treatment.	Last blood count after treatment.	Change.	Interval between counts.
					per cent	*days*
13	Yali.....	2 x 3.0 gm. 14.0 gm.	R.B.C. 3,360,000	4,800,000	+ 43	88
	51.5 kg.	2 x 4.0 "	Hb. 45%	75%	+ 67	
		272 mg. per kg. in 21 days.	W.B.C. 2,300	8,000	+248	
1	Eali.....	2 x 2.0 gm. 4.0 gm.	R.B.C. 2,720,000	4,160,000	+ 49	20*
	57.0 kg.	70 mg. per kg. in 13 days.	Hb. 35%	51%	+ 46	
			W.B.C. 4,400	9,000	+104	
2	Bibaba.....	4 x 3.0 gm. 12.0 gm.	R.B.C. 2,480,000	3,616,000	+ 46	35*
	59.0 kg.	203 mg. per kg. in 9 days.	Hb. 48%	55%	+ 14	
			W.B.C. 3,500	7,000	+100	
3	M'Boyo.....	4 x 3.0 gm. 12.0 gm.	R.B.C. 2,780,000	3,680,000	+ 32	99
	49.0 kg.	in 9 days.	Hb. 35%	60%	+ 71	
		3 x 4.0 gm. 12.0 gm.	W.B.C. 2,700	6,000	+122	
		in 14 days.				
		24.0 gm. 490 mg. per kg.				
4	Etumbu.....	3 x 2.0 gm. 9.0 gm.	R.B.C. 2,280,000	2,480,000	+ 9	24†
	48.5 kg.	1 x 3.0 "	Hb. 34%	40%	+ 18	
		185 mg. per kg. in 9 days.	W.B.C. 9,500	12,580	+ 32	

21	Monololo..... 60.0 kg.	4 x 4.0 gm. in 21 days.	16.0 gm.	R.B.C.	4,800,000	5,120,000	+ 7	84
				Hb.	70%	75%	+ 7	
		2 x 6.0 gm.	20.0 gm.	W.B.C.	8,000	8,000	—	
		2 x 4.0 "						
		36.0 gm.	600 mg. per kg.					
27	K. Lukas..... 48.0 kg.	3 x 5.0 gm. in 14 days.	15.0 gm.	R.B.C.	4,000,000	4,600,000	+ 15	79
				Hb.	60%	74%	+ 25	
		8 x 2.0 gm. in 26 days.	16.0 gm.	W.B.C.	4,000	7,000	+ 75	
		31.0 gm.	646 mg. per kg.					

* Trypanosomiasis exacerbation (?).

† Acute amœbiosis; trypanosomiasis exacerbation (?).

mide treatment in practically every patient, with the exception of those who had complicating infections and those who presented the only untoward effect observed. This consisted in the occurrence of a visual impairment in 9 advanced cases.

TABLE XXIX.

Changes in the Red Blood Cells of Advanced Cases Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	Total amount tryparsamide.	R. B. C. before treatment	Last R. B. C. after treatment.	Change.	Interval between counts.
		mg. per kg.			*per cent*	*days*
18	Loyoko	150	2,720,000	4,800,000	+ 76	82
35	Koloshi	449	3,840,000	5,120,000	+ 33	45
29	Fulu	314	4,160,000	4,160,000	—	46
32	N'Siola	400	4,160,000	5,120,000	+ 23	73
49	Makalamba	171	4,320,000	4,640,000	+ 7	47
17	Bwaba	427	2,400,000	4,060,000	+ 69	87
22	Bose	464	2,560,000	5,120,000	+100	85
9	Bolendo	449	2,912,000	4,000,000	+ 37	88
10	Makengo	541	2,940,000	4,480,000	+ 52	88
16	Masimango	458	3,040,000	5,040,000	+ 66	84
24	Miaku	411	3,104,000	3,200,000	+ 3	78
14	Tono	460	3,352,000	4,000,000	+ 19	81
6	Nembezi	568	3,616,000	4,160,000	+ 15	93
25	Madina	583	3,728,000	4,000,000	+ 7	74
15	Babuli	452	3,840,000	4,320,000	+ 13	81
20	Kiale	680	4,000,000	4,320,000	+ 8	81
26	M'Pala	468	4,136,000	5,120,000	+ 24	71
4	Etumbu	185	2,280,000	2,480,000	+ 9	24
2	Bibaba	203	2,480,000	3,616,000	+ 46	35
1	Eali	70	2,720,000	4,160,000	+ 49	20
3	M'Boyo	490	2,780,000	3,680,000	+ 32	99
13	Yali	272	3,360,000	4,800,000	+ 43	88
22	K. Lukas	646	4,000,000	4,600,000	+ 15	79
21	Monololo	600	4,800,000	5,120,000	+ 7	84

The first 5 patients were treated with 1 course of tryparsamide; the next 12 with 2 courses; the last 7 had been previously treated with other drugs.

Before discussing the significant features of this disturbance, a brief description of its occurrence in each patient will be given. The first 5 patients were known to have received previous treatment with

arsenical drugs and we suspected that one other, Miaku #24, had been treated before. All had marked involvement of the cerebrospinal system on admittance as shown clinically and by spinal fluid examination with the exception of Monololo #21.

TABLE XXX.

Changes in the Hemoglobin Content of the Blood of Advanced Cases Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	Total amount tryparsamide.	Hemoglobin before treatment.	Hemoglobin after treatment.	Change.	Interval between counts.
		mg. per kg.			*per cent*	*days*
18	Loyoko	150	45	80	+78	82
35	Koloshi	449	45	60	+34	45
29	Fulu	314	55	50	− 9	46
32	N'Siola	400	60	75	+25	73
49	Makalamba	171	70	75	+ 7	47
6	Nembezi	568	40	65	+63	93
17	Bwaba	427	42	55	+31	87
15	Babuli	452	46	65	+41	81
14	Tono	460	48	75	+56	81
10	Makengo	541	48	60	+25	88
16	Masimango	458	50	70	+40	84
25	Madina	583	50	70	+40	74
22	Bose	464	52	75	+44	85
24	Miaku	411	52	65	+25	78
26	M'Pala	468	55	80	+45	71
9	Bolendo	449	55	65	+18	88
20	Kiale	680	60	70	+17	81
4	Etumbu	185	34	40	+18	24
1	Eali	70	35	51	+46	20
3	M'Boyo	490	35	60	+71	99
13	Yali	272	45	75	+67	88
2	Bibaba	203	48	55	+14	35
27	K. Lukas	646	60	75	+25	79
21	Monololo	600	70	75	+ 7	84

The first 5 patients were treated with 1 course of tryparsamide; the next 12 with 2 courses; the last 7 had been previously treated with other drugs.

Eali # 1.—Previous treatment, 0.75 gm. atoxyl. Lumbar puncture, 80 lymphocytes per cmm. Slightly exaggerated deep reflexes; mental condition normal. He was given 3 doses of 2.0 gm. in 27 days, followed after a week's interval by 2 doses of 4.0 gm. in 7 days. 2 days after the last dose, he complained of indis-

tinctness of vision of both eyes; walked unsteadily but could count fingers; treatment stopped. A month later he could count fingers with left eye but not with right; he could distinguish light with both General condition became worse, with fever, etc. (exacerbation of trypanosomiasis?), and death occurred.

TABLE XXXI.

Changes in the White Blood Cells of Advanced Cases Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	Total amount tryparsamide.	W. B. C. before treatment.	Last W. B. C. after treatment.	Change.	Interval between counts.
		mg. per kg.			*per cent*	*days*
29	Fulu	314	4,500	11,000	+144	46
35	Koloshi	449	5,000	8,000	+ 60	45
18	Loyoko	150	7,500	8,000	+ 7	82
32	N'Siola	400	8,500	8,000	− 6	73
49	Makalamba	171	11,000	10,000	− 9	47
15	Babuli	452	4,000	8,000	+100	81
22	Bose	464	5,200	8,400	+ 61	85
24	Miaku	411	5,400	4,400	− 18	78
25	Madina	583	5,550	7,800	+ 40	74
14	Tono	460	6,000	5,200	− 13	81
9	Bolendo	449	6,200	7,000	+ 13	88
16	Masimango	458	7,500	9,000	+ 20	84
6	Nembezi	568	8,500	6,600	− 22	93
26	M'Pala	468	9,600	10,000	+ 4	71
10	Makengo	541	10,000	7,200	− 28	88
17	Bwaba	427	12,000	8,000	− 33	87
20	Kiale	680	12,000	9,000	− 25	81
13	Yali	272	2,300	8,000	+248	88
3	M'Boyo	490	2,700	6,000	+122	99
2	Bibaba	203	3,500	7,000	+100	35
27	K. Lukas	646	4,000	7,000	+ 75	79
1	Eali	70	4,400	9,000	+104	20
21	Monololo	600	8,000	8,000	−	84
4	Etumbu	185	9,500	12,580	+ 32	24

The first 5 patients were treated with 1 course of tryparsamide; the next 12 with 2 courses; the last 7 had been previously treated with other drugs.

Bibaba № 2.—Previous treatment: soamin, 5 doses of 0.5 gm., and tartar emetic, 4 doses of 0.1 gm., during 43 days. Lumbar puncture 37.5 lymphocytes per cmm. Greatly exaggerated deep reflexes and mental apathy. He was given 4 doses of 3.0 gm. or 203 mg. per kilo in 9 days. 2 days after last injection, complained of

TABLE XXXII.

Weight Changes of Advanced Cases (No Previous Treatment) Treated with Repeated Doses of Tryparsamide.

Case No.	Name.	Spinal fluid before treatment.	Doses of tryparsamide.	Initial weight.	2nd week.	4th week.	6th week.	8th week.	10th week.	12th week.	14th week.	Total change.
			gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
18	Loyoko.....	17.5	3 x 3.0	60	60	63	62		61.5	60		0‡‡
49	Makalamba...	22.0	9 x 1.0	52.5	54.5	54	53	53.5				+1.0§§
9	Bolendo.....	47.5	5 x 2.0 3 x 4.0	49	51	51	53		56.5	57	57.5	+8.5
29	Fulu.....	142.5	8 x 1.0 1 x 2.0 2 x 1.0	25.5	25	25.5	25	26	25.5			0**
32	N'Siola.....	52.5	4 x 4.0	40	39.5	40	38.5	39	41			+1.0
35	Koloshi.....	285.0	4 x 5.0	44.5	44.5	45	44.5	44.5	45			+0.5††
44	N'Kusu.....	47.5	4 x 4.0 2 x 2.0	38.5	37.5	39	39	39	39.5			+1.0
22	Bose.....	15	2 x 3.0 4 x 5.0	56	55	56	56	58.5	59.5	60.5		+4.5
26	M'Pala.....	15	3 x 3.0 4 x 5.0	62	60.5	61.5	64	62	63	63		+1.0‡
6	Nembezi.....	+(?)	5 x 2.0 4 x 4.0	46	47.5	47.5	48.5		51	51	52	+6.0‖‖
14	Tono.....	20.2	4 x 2.0 1 x 0.7 3 x 4.0	45	48	47	48	49	48.5	49		+4.0
16	Masimango...	35	5 x 2.0 3 x 4.0	48	47.5	47.5	48	48	48.5	48.5		+0.5
15	Babuli.....	70	5 x 2.0 4 x 4.0	57.5	58	55	56	56.5	57	57		−0.5§

TABLE XXXII.—*Concluded.*

Case No.	Name.	Spinal fluid before treatment.	Doses of tryparsamide.	Initial weight.	2nd week.	4th week.	6th week.	8th week.	10th week.	12th week.	14th week.	Total change.
			gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
24	Miaku*.....	305	2 x 4.0 5 x 2.0	46.5	47.5	46.5	†	†		47.5		+1.0‖
25	Madina.....	300	3 x 4.0 8 x 2.0	48	47.5	47.5	48	50.5	51	51		+3.0‡
20	Kiale*.....	387.5	4 x 4.0 8 x 2.0	47	48.5	50	50.5	50	51	51		+4.0***
17	Bwaba.....	450	4 x 3.0 5 x 2.0	51.5	52	49.5		47		45		−6.5‖
10	Makengo.....	247.5	1 x 1.0 1 x 2.0 1 x 3.0 1 x 4.0 1 x 5.0 1 x 2.0 1 x 0.5 5 x 1.0	41.5	43	42	41	45.5	46.5	48	49	+7.5

* Previous treatment suspected.
† In bed.
‡ Malaria.
§ Malaria; leg abscess.
‖ Trypanosomiasis exacerbation (?).
** Pustular skin infection; filariasis; scabies.
†† Chronic amœbic dysentery; ankylostomiasis.
‡‡ Filariasis.
§§ Syphilis; malaria.
‖‖ Malaria (?).
*** Tinea versicolor.

indistinctness of vision—worse in left than right eye; could distinguish light with both eyes. Condition improved during following 2 weeks so that patient could count fingers with both eyes but general condition became much worse with fever, etc. (exacerbation of trypanosomiasis?). 2 doses of 2.0 gm. were given with no increase in visual impairment but without influence upon the course of the disease and death occurred.

TABLE XXXIII.

Weight Changes of Advanced Previously Treated Cases After Repeated Doses of Tryparsamide.

Case No.	Name.	Spinal fluid before treatment.	Doses of tryparsamide.	Initial weight.	2nd week.	4th week.	6th week.	8th week.	10th week.	12th week.	14th week.	16th week.	Total change.
			gm.	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*	*kg.*
13	Yali.....	32	2 x 3.0 2 x 4.0	51.5	52.5	51.5	51	51.5	54	54	54.5		+3.0
1	Eali.....	80	2 x 2.0 1 x 2.0 2 x 4.0	57	56	58	56	55					−2.0*
2	Bibaba....	37.5	4 x 3.0 2 x 2.0	59	59		52						−7.0*
3	M'Boyo...	92.5	4 x 3.0 3 x 4.0	49	50	51	52.5		53.5		54.5	56	+7.0
4	Etumbu..	142.5	3 x 2.0 1 x 3.0	48.5	49	44							−4.5†
21	Monololo.	37.5	4 x 4.0 2 x 6.0 2 x 4.0 1 x 3.0	60	60	62	61.5	62	62	63			+3.0
27	K. Lukas.	327.5	3 x 5.0 8 x 2.0	48	47	48	50.5	50	52.5	52.5			+4.5
7	Engondo..	572.0	3 x 4.0 6 x 2.0	45							49		+4.0
8	Tokamba.	100.0	1 x 4.0 8 x 2.0 6 x 2.0	39		43	42.5				44		+5.0
34	Langomo.	467.5	3 x 7.0	62.5	62	63	63						+0.5

* Died. Trypanosomiasis exacerbation (?).

† Died. Amœbic dysentery; trypanosomiasis exacerbation (?).

M'Boyo # 3.—Previous treatment: soamin, 4 doses of 0.5 gm., and tartar emetic, 2 doses of 0.1 gm., in 26 days. Lumbar puncture 92.5 lymphocytes per cmm.; large mononuclears +++. Mental deterioration, apathy, and lethargy; marked nervous symptoms. She was given 4 doses of 3.0 gm. or 245 mg. per kilo in 9 days. 2 to 3 days after last dose, she complained of dimness of vision in both eyes and she walked unsteadily; there was complete recovery in 18 days.

Treatment was resumed after 3 weeks' intermission with 3 weekly doses of 4.0 gm. so that the same total amount of tryparsamide was given but in 14 instead of 9 days. There were no subsequent disturbances. Ophthalmoscopic examination 2 months after the last dose of tryparsamide was negative except for a slight reduction in size of the optic discs.

Etumbu # 4.—Previous treatment: soamin, 3 doses of 0.5 gm., and tartar emetic, 2 doses of 0.1 gm. in 22 days. Lumbar puncture 142.5 lymphocytes per cmm.; large mononuclears +. Mental apathy. He was given 3 doses of 2.0 gm. and 1 dose of 3.0 gm. or 185 mg. per kilo in 9 days. 3 to 4 days after the last dose he complained of indistinctness of vision of both eyes and treatment was stopped; the sight became normal in about 2 weeks.

Monololo # 21.—Previous treatment unknown. Lumbar puncture 37.5 lymphocytes per cmm. No mental or nervous symptoms. He was given 4 weekly doses of 4.0 gm. or 267 mg. per kilo in 21 days with no reaction of any sort. Treatment was resumed 2 weeks later and 2 doses of 6.0 gm. were given 10 days apart. A week later he complained of slight indistinctness of vision of both eyes. At his next visit, 11 days later, vision of left eye was normal and a week later vision of both eyes was normal.

Subsequent treatment with 2 doses of 4.0 gm. and 1 dose of 3.0 gm. in 19 days caused no visual disturbance whatever.

Miaku # 24.—Previous treatment suspected. Lumbar puncture 305 lymphocytes per cmm.; large mononuclears++; mulberry cells++. Mentally apathetic, beginning lethargy (?). Marked nervous symptoms. He was given 2 weekly doses of 4.0 gm. or 172 mg. per kilo in 7 days. Complained of indistinctness of vision of both eyes and intense headache a week later. Could count fingers and gait was normal. Treatment stopped. 2 days later, vision of left eye normal; right eye slightly improved. 3 weeks later, patient became very ill with fever, etc. (exacerbation of trypanosomiasis?), and he was given 5 weekly doses of 2.0 gm. with marked clinical improvement and no increase of the residual visual impairment of the right eye.

At the end of this course of tryparsamide, vision of the left eye was normal. With the right eye he could distinguish light but vision was poor. Ophthalmoscopic examination of both eye grounds showed a pale retina with clearly defined, somewhat pale optic discs but otherwise no abnormality.

Bwaba # 17.—No previous treatment. Lumbar puncture 450 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. Mental deterioration; marked nervous symptoms. He was given 4 weekly doses of 3.0 gm. or 233 mg. per kilo in 21 days. On the last day of treatment, he complained of conjunctivitis

of the right eye with pericorneal injection and pustular discharge; no urethral discharge. Condition responded promptly to local treatment. A week later, complained of right eye being "bad" and in 2 days there was marked visual impairment of both eyes; could count fingers and see to walk.

During the following month, vision of both eyes improved slightly. Treatment with weekly doses of 2.0 gm. was resumed after the appearance of a brawny indurated swelling of the left leg and foot (trypanosomiasis?) and no intensification of visual symptoms was observed.

2 months after the onset of visual impairment, opthalmosocopic examination of both eyes showed an anemic retina with a flattened white optic disc, the vessels of which were sharply outlined and distinctly seen. Size of disc apparently enlarged. Vision of left eye poor—of right, slight; could see to walk.

Makengo # 10.—No previous treatment. Lumbar puncture 247.5 lymphocytes per cmm.; large mononuclears ++; mulberry cells ++. Dull mental condition, slight lethargy, marked nervous symptoms. He was given increasing doses of tryparsamide from 1.0 to 5.0 gm., a total of 15.0 gm. or 361 mg. per kilo in 24 days. 5 days after the last dose, he complained of visual dimness of right eye; could not count fingers with this eye. Vision of left eye unimpaired. During the following month, the sight improved considerably. He could see objects but stated that there was a "shadow" over them. Treatment was resumed with small doses (2.0, 0.5, and 1.0 gm.) with no increase in visual symptoms. A month later, there was a slight visual dimness of the right eye which was gradually decreasing and the left eye was normal. Ophthalmoscopic examination of both eye grounds showed a pale optic disc, the vessels of which were distinctly visible, and a pale retina with small vessels.

Bandu # 54.—No previous treatment. Good general physical condition; 71 kilos. Marked edema of both legs from knee to toes. No mental or nervous symptoms. Lumbar puncture 117 lymphocytes per cmm.; large mononuclears +. 3 mulberry cells seen. He was treated at weekly intervals with 5.0 and 6.0 gm; and 4 doses of 2.0 gm., a total of 19.0 gm. or 290 mg. per kilo in 26 days. 3 days after the last dose there was slight unsteadiness and dimness of vision of both eyes; no edema except over instep of both feet; urine examinations negative. 2 weeks later, vision of both eyes was normal; ophthalmoscopic examination showed normal retinæ and slightly enlarged, pale discs. The pronounced edema of both legs had entirely disappeared by this time and a lumbar puncture 8 weeks after admittance showed a normal spinal fluid.

These observations may be summarized as follows: There were 17 instances of visual impairment of which 6 were slight, 7 were moderate, and 4 of marked degree. In 10, recovery was complete and 3 showed moderate and 4 slight improvement.

From an analysis of the above cases, certain factors in regard to the occurrence of visual impairment should be pointed out. First, all

were advanced cases, with marked alteration of the spinal fluid except 2, Monololo #21 and Bibaba #2. No visual disturbance of any kind occurred in patients in the early stages of the disease or in the beginning of the advanced period, irrespective of the system of therapy employed.

Next, 4 of the 9 patients were being treated with atoxyl and tartar emetic at the time the administration of tryparsamide was begun, while a fifth patient, and we suspected a sixth, had had previous arsenical treatment. To what extent previous treatment may contribute to the production of visual impairment is uncertain but that such a drug as atoxyl for instance may participate in this reaction is not improbable.

In the third place, there was no instance of sudden complete blindness but the condition was one of visual dimness of various grades or of inability to see distinctly; in every instance, there was either complete recovery or improvement of some degree. It is of interest to note that a marked and moderate impairment of 2 patients which was similar in degree to that observed in others was entirely transitory.

Finally as regards tryparsamide itself, it should be pointed out that no single dose of even 7.0 gm., given in this instance to a very advanced case (Langomo #34), produced untoward effects of any kind. In one patient with a markedly abnormal spinal fluid, 2 doses of 4.0 gm. separated by an interval of a week, were followed by visual disturbances but in the others more than 2 doses were given. Attention should also be drawn to the fact that in several patients administration of considerable amounts of the drug, both before and after the occurrence of visual impairment, did not reproduce this effect or if any residual symptoms were present at the time of retreatment they were not increased by further drug administration. For example, M'Boyo #3, who had completely recovered from the disturbance which followed the first course of 12.0 gm., was afterwards given exactly the same amount of drug, 3 doses of 4.0 gm. over a slightly longer period of time, with no visual reaction whatever, and Makengo #10 who had partially recovered was given a second course of treatment of smaller doses with no increase of visual impairment.

The size of the dose together with the interval of time between doses are undoubtedly crucial factors and of the two the spacing of the doses

is probably the more important. Bibaba #2, M'Boyo #3, and Etumbu #4 were given 4 doses of 2.0 and 3.0 gm. at 2 and 3 day intervals; all of these patients developed a considerable degree of visual impairment but it was entirely transitory with M'Boyo #3 and Etumbu #4, while Bibaba #2 showed a moderate improvement. These cases illustrate the danger of too frequent dosage. The case of Makengo #10 is somewhat analogous. He was given ascending doses of from 1.0 to 5.0 gm. in 24 days with no untoward effects until a few days after the last dose. Undoubtedly with this type of treatment, the larger doses were given too closely together and it is probable that if the last dose of 5.0 gm. had been omitted no visual symptoms would have occurred. On the other hand, 3 other patients who were given weekly doses of 3.0 and 4.0 gm. developed visual impairment, Eali #1, Miaku #24, and Bwaba #17. In contrast with these cases, it should be recalled that other advanced patients who were given similar or larger weekly doses showed no resultant visual symptoms. For instance, Koloshi #35 was given 4 doses of 5.0 gm. in 29 days; K. Lukas #27, 3 doses of 5.0 gm. in 14 days; and Langomo #34, 3 doses of 7.0 gm. in 21 days, as shown in Tables XXIV to XXVI. It seems not unlikely, therefore, that the individual pathological condition of the optic structures in advanced cases is the principal predisposing factor in the occurrence of this untoward effect.

DISCUSSION.

The purpose of the work reported in this paper was the obtaining of certain fundamental facts in regard to the action of tryparsamide in human trypanosomiasis since experimental toxicologic and therapeutic investigations with the drug had indicated its possible use in the treatment of patients suffering from this disease. The observations of 77 patients in early and advanced stages of trypanosomiasis caused by *Tr. gambiense* and treated with tryparsamide formed the basis for this study. The patients came from various parts of the Belgian Congo and most of them had not received previous treatment.

It was realized that objective information on the action of single doses must precede the use of several doses and, further, that the effect of a few doses must be studied before one could devise a logical system of repeated dose therapy. Consequently the work was conducted

along the general lines suggested by these principles. The first observations were concerned with determining the trypanocidal action of various sized doses of tryparsamide by microscopic examinations of centrifuged blood and lymph gland juice and included both the time of occurrence of this action and its duration in relation to the time of treatment. From a series of some 90 examinations after the treatment of patients in all stages of the disease with doses ranging from 0.3 to 7.0 gm. or 4.7 to 112 mg. per kilo of body weight, administered intravenously in most instances, it was found that tryparsamide was an active trypanocidal agent since its administration was consistently followed by negative examinations for trypanosomes in the superficial lymph glands and blood within 24 hours. The smaller doses up to 50 mg. per kilo of body weight produced negative lymph gland and blood examinations within 15 hours in the majority of cases and this number was increased after larger doses of 54 to 112 mg. per kilo In certain patients, no trypanosomes were found in the blood as early as $3\frac{1}{2}$ hours after treatment but, in general, doses of 2.0, 3.0, and 4.0 gm. produced a peripheral sterilization in from 6 to 12 hours.

The duration of peripheral sterilization induced by single doses of tryparsamide was next studied in patients in the first period of the disease or as indicated by a slight increase in the number of cells of the cerebrospinal fluid, in the beginning of the advanced period. Without taking into account differences in the severity or type of the infections in individual patients, it was found that the duration of peripheral sterilization varied from 17 to 58 days in the 12 patients who had been given single doses of 0.5 to 5.0 gm. or from 17 to 83 mg. per kilo of body weight. On the other hand, no peripheral relapse was detected during observation periods of from 40 to 111 days in the 9 patients who had been treated with single doses of 0.5 to 3.0 gm. or from 9 to 68 mg. per kilo. It appears, therefore, that within the limits of dosage of from 0.5 to 5.0 gm., peripheral sterilization in relapsed patients endures on an average of from 3 to 6 weeks; but since there was one relapse 17 days after treatment, it seemed advisable in planning a system of repeated doses to begin with intervals of 1 and 2 weeks until the results of larger series of single dose administrations should be available for consideration.

The effects of intramuscular administration upon the duration of peripheral sterilization is significant. 4 patients were treated by this route with single doses of from 37 to 57 mg. per kilo; the immediate effect upon the trypanosomes in lymph glands and blood was similar to that observed after the intravenous route but on the whole the duration of this action was definitely more prolonged as seen by the average time of relapse, 7 weeks after treatment. In 3 patients, there was practically no local reaction while the tenderness and induration which occurred in one instance was probably due to a passing mild infection. While no subcutaneous injections were given, a slight amount of drug solution escaped into the tissues surrounding the vein in a few instances, but no irritation or discomfort of any sort arose, and in a few hours complete absorption had taken place.

The spinal fluid of early cases treated with single doses of tryparsamide was examined because of the possibility that the disease might progress and involve the central nervous system during the period of blood and lymph gland sterilization. A lumbar puncture was made on an average of 5 weeks after treatment in 14 patients with initial normal or slightly abnormal spinal fluids who had been given single doses ranging from 0.5 to 3.0 gm. or 9.0 to 68.0 mg. per kilo. In every instance, except one, the second cell count continued to be normal or was decreased to within or toward normal limits; in one patient, the result was uncertain because of blood contamination of the first specimen of spinal fluid. No trypanosomes, however, could be found in the lymph gland juice, centrifuged blood, or centrifuged spinal fluid of this patient.

The general physical reaction of patients in the first period of the disease treated with single doses was prompt and satisfactory. In 2 or 3 days after treatment, the subjective symptoms disappeared and the pulse rate and temperature became normal; within a month the blood count was materially improved, the patient had gained weight and the cervical lymph glands were small and indurated. Interruptions of this favorable course of events occurred in those patients who harbored other infections or who ultimately had a trypanosomal relapse but in general a definite and usually a marked beneficial effect was observed. In addition, no untoward reactions of any kind

were observed in these patients, either of an immediate occurrence or of a more delayed appearance.

The use of repeated doses of tryparsamide was based upon the action of single doses, and a number of previously untreated patients as well as those who had suffered a relapse after single doses were given from 4 to 7 doses of 2.0 to 5.0 gm. at weekly or fortnightly intervals. Only the initial observations are at present available. The preliminary effect as regards negative trypanosome examinations of lymph glands and blood and general clinical improvement indicated by the disappearance of subjective symptoms, pulse and temperature curves, blood counts, gain in strength, and increase in weight occurred in both groups of patients. Microscopic examinations of lymph gland juice and centrifuged blood made from time to time during the course of treatment were uniformly negative.

Obviously no predictions of the ultimate therapeutic results in these patients can be made, and while it is not unlikely that future experience may show that a more prolonged system of therapy is necessary, it seemed advisable in the beginning to determine the effect of a relatively small number of doses.

The treatment of advanced cases of trypanosomiasis must take into account not only the invasion of the cerebrospinal axis by the infecting organism and the resultant injuries but also the chronicity of the disease, its tendency toward exacerbation and the debilitated physical state of many patients. With these facts in view, some system of repeated dose therapy appears obligatory and the treatment with tryparsamide, therefore, based upon the action of single doses, consisted in the administration of several doses ranging from 1.0 to to 7.0 gm. at intervals of from 3 to 14 days. 28 patients who presented a wide variety of nervous and mental symptoms and whose spinal fluid showed an increased cell content of from 15 to 572 lymphocytes per cmm., together with large mononuclears and mulberry cells in most instances, were under observation for a sufficient length of time after treatment to permit of subsequent lumbar punctures as well as other clinical examination. The cell content of the spinal fluid was used as a criterion of the degree of cerebrospinal involvement as well as a therapeutic guide and also as an index of the effect of treatment.

The immediate sterilizing action of tryparsamide was noted in those patients whose lymph gland examination was positive on admittance; trypanosomes were not found in 7 patients who were being treated with atoxyl and tartar emetic at the time of transfer to tryparsamide therapy. Repeated microscopic examinations of lymph gland juice and centrifuged blood made during the period of observation were negative in all patients. In general, the physical status of the patients was materially benefited as was shown by the return of the pulse and temperature to normal limits, the prompt disappearance of the subjective symptoms and by improved blood counts and gains in weight. The improvement in habits and personal appearance of several patients was also noticeable. The early termination of treatment in 3 patients was followed by a clinical exacerbation of the disease and ensuing death; in one of the 3 there was a complicating acute amœbic dysentery. The nervous and mental symptoms which were present in many instances were greatly improved or completely eliminated except in 2 very advanced patients with marked mental deterioration who had been treated with other drugs for long periods of time and in whom the change was slight.

The effect of tryparsamide upon advanced trypanosomiasis as illustrated by this group of patients is further evidenced by the marked and rapid diminution of the cells of the spinal fluid. In general, 3 or 4 weekly doses of 3.0, 4.0, or 5.0 gm. produced decreases of from 57 to 98 per cent in an average of 5 weeks. In 5 patients whose treatment was limited to one such course, the cell content continued to decrease in 4 instances as shown by a practically normal third examination made 9 to 12 weeks after admittance, while in 1 patient, there was no further decrease. 16 patients who were given second courses of treatment showed a continued decrease of cells; 3 showed practically no change from the count made after the first course of treatment; in one, there was a slight increase in cells and in another, after 3 irregularly spaced doses, the cells had returned to practically the original count. In this limited number of cases, it was apparently more difficult to accomplish a further decrease of the spinal fluid cell content by a second course of treatment than it was to produce the initial and marked diminution by the first course, but at any rate, in these 5 instances, the active progression of the disease was apparently

arrested. Considering the pathological conditions of cerebrospinal trypanosomiasis, it seems not unlikely that a longer first course of treatment than that employed in most of these patients would give more satisfactory results. Regarding the effect produced by more frequent smaller doses as contrasted with larger doses separated by longer intervals of time, it appears that the use of semi-weekly doses of 2.0 gm. for instance had apparently no advantage over a weekly dose of 3.0 or 4.0 gm.

Finally, we wish to consider the only untoward effect encountered during the course of this work. Dimness of vision of various degrees occurred in 9 advanced cases, 5 of whom had received previous arsenical treatment. It should be pointed out that there was no instance of visual disturbance of any kind in the early cases or in the first stages of the cerebrospinal period of the disease or after any single dose of tryparsamide. The largest dose given in our cases was 7.0 gm. In the majority of instances, this effect was entirely transitory and in the others various degrees of improvement took place. 3 patients who were given too frequent an administration of medium sized doses showed considerable degrees of impairment and of these 2 recovered completely; of 3 other patients with about the same degree of impairment one showed slight improvement, another moderate improvement and the third complete recovery, while in the remaining 3 a less marked visual disability was transitory. It would seem from a study of these cases that too frequent administration of medium and large doses to patients suffering from very advanced trypanosomiasis is one factor that contributed to this condition. It is significant to note in this connection that resumption of treatment with smaller or more widely spaced doses was not followed by a return of the condition or by an intensification of residual symptoms. In addition, the time of occurrence of this untoward effect, 2 to 7 days after treatment, together with the nature of the pathological injury to the optic structures in cerebrospinal trypanosomiasis and the fact that improvement of some degree always occurred, suggests that the cause of the visual impairment is related to the processes of resolution and healing of the lesion already present rather than to a direct toxic action upon the nerve itself. The ophthalmoscopic examination of several patients who had partially or completely

recovered from this condition also lends support to this view. The experimental work with tryparsamide showed that apparently the drug possesses the power of tissue penetrability to a marked degree since a characteristic feature of the treatment of rabbit trypanosomiasis was the rapidity and completeness with which extensive cutaneous and subcutaneous lesions regressed and healed. Individual variations in the extent or degree of lesions is the probable explanation of the fact that several patients with as markedly altered spinal fluids and as pronounced nervous and mental symptoms developed no visual disturbances whatever after similar treatment.

From the facts at our disposal, it would appear that the occasional occurrence of visual disturbances, which may perhaps be avoided by a slightly different system of dose repetition, does not at present constitute an objection to the use of the drug. The impression which one has after treating advanced cases with tryparsamide is that a suitable interval between doses of a certain size is probably the most important factor in preventing the occurrence of untoward visual effects. Doses of 3.0 gm. or over should preferably not be given oftener than once a week, and since longer intervals between doses have been followed by clinical improvement and marked decreases in the cells of the spinal fluid, further use of a system of wider spaced repeated doses may show similar beneficial therapeutic effects without concomitant visual disturbances.

SUMMARY.

The present study of the action of tryparsamide in human trypanosomiasis concludes a series of chemical and biological investigations in a particular problem of chemotherapy and thus represents the final step in a logical method of approach to such a problem. It has been shown that tryparsamide, the sodium salt of *N*-phenylglycineamide-*p*-arsonic acid, possesses a marked trypanocidal activity in human trypanosomiasis caused by *Tr. gambiense*. Single doses of from 0.5 to 5.0 gm. produced a peripheral sterilization of lymph glands and blood in an average of 6 to 12 hours. The duration of the peripheral sterilization following single doses of 17 to 83 mg. per kilo ranged from 17 to 58 days in patients who ultimately showed a return of trypanosomes to the peripheral blood. In a number of patients, however,

treated with single doses of 9 to 68 mg. per kilo, no such relapse was detected during an observation period of from 40 to 111 days. The drug is extremely soluble in water and may be administered intramuscularly as well as intravenously. The immediate trypanocidal action after intramuscular administration was as rapid as that following the intravenous route while the duration of peripheral sterilization was appreciably longer.

Relatively few repeated doses produced in advanced cases a marked and rapid diminution of the cells of the spinal fluid and were associated with definite improvement of mental and nervous symptoms. The occurrence of visual disturbances in certain advanced cases was the only untoward effect detected during the course of the work, and was apparently related to a too frequent administration of the drug. The condition was transitory in the majority of instances and resumption of treatment was not followed by a recurrence of this symptom.

The general beneficial effect of the drug was a noticeable feature of its action in both early and advanced cases as shown by the disappearance of subjective symptoms, by the return of the pulse and temperature to normal limits, by the pronounced improvement of the blood picture, and by well marked gains in weight.

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ON THE NATURE OF THE SO CALLED GERM CENTER IN LYMPHOID TISSUE.

By WARO NAKAHARA and JAMES B. MURPHY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 1.

INTRODUCTION.

The lymphopoietic function of the spleen and lymph-nodes is a well-known fact, but as to just how the production of new lymphocytes is brought about in these organs is still a matter of uncertainty. Flemming ('85) first called attention to the frequent occurrence of mitosis in the tissue of lymphoid organs, especially at a certain spot in the follicle. To this spot he gave the name 'germ center,' believing it to be a birth-place of new lymphocytes. The view has been traditionally followed in numerous texts, in which the lymphoblastic nature of the cells of the germ center is generally accepted and is based entirely on the interpretation of the static 'picture' as observed in preserved material. On account of the obvious limitations of this method of observation, the deductions made on it lack finality.

In the course of experiments on various types of general lymphoid reaction, we have collected observations which may serve to throw additional light on the process. A résumé of the observations and a brief discussion bearing on them are given in this paper in the hope of elucidating somewhat the point under consideration.

OBSERVATION.

Lymphoid Reaction Induced by Dry Heat.

It was reported by Murphy and Sturm ('19) that animals subjected to a small exposure of dry heat (using an electric heating lamp as the source of heat) show, following a sharp initial fall, a rise of circulating lymphocytes often amounting to more than 200 to 300 per cent above the normal. Polymorphonuclear leucocytes partici-

pate in the fall, but they recover their normal level slowly and never rise above it.

Histological examination (Nakahara, '19) of the spleen and lymph-nodes of animals carried as a parallel to the above experiment showed that these organs, immediately after the heat treatment, contain numerous necrotic cells. The dead cells were present abundantly in the nodules and in the pulp of spleen, cortex, and lymph-cords of nodes. The cells of the germ centers were apparently unaffected, but cell division was totally suppressed.

It was also observed at about forty-eight hours after the treatment that, along with an elimination of the necrotic cells, there was an abnormally large number of mitotic figures present in the germ centers, and this hyperactivity of the centers continued for several days.

Lymphoid Reaction in Immunity to Transplated Cancer Induced by an Injection of Homologous Living Tissue.

Mice, as is well known, can be made relatively resistant to transplanted cancer by injecting them with an emulsion of homologous living tissue ten days before inoculating the cancer grafts. Murphy and Morton ('15) found that the lymphocytic elements of the blood showed an active increase during this process, while polymorphonuclear cells were not appreciably affected. No change in the lymphocytes occurred in the resistant mice until the cancer graft was introduced, when an immediate and sharp rise took place in these cells. In the majority of animals the increase was 100 to 200 per cent above the normal and endured about two weeks.

The histological study (Murphy and Nakahara, '20) made of preserved material from the inoculated and resistant mice showed in the germ centers of the lymphoid organs increase in mitotic figures, reaching a maximum about five days after the immunizing injection and followed by a fall, the normal being regained by the tenth day. The resistant mice when inoculated with a cancer graft ten days after the tissue injection show a second great increase in the mitotic figures, particularly of the germ centers. Judging from the number of mitosis, this second stimulation is more intense than the first.

Lymphoid Reaction Induced by Small Doses of X-Rays.

The selective action of X-rays on the lymphoid tissue has been recognized since the work of Heineke ('05). It was shown in this laboratory that while large doses of x-ray are destructive to, small doses bring about an increase in the lymphoid cells (Murphy and Morton, '15). In the case of rabbits thus treated, the number of circulating lymphocytes rose often in the course of one week (Thomas Taylor, and Witherbee, '19) 100 per cent above the normal.

Histologically (Nakahara, '19) in these animals no appreciable destruction of cellular elements of spleen and lymph-nodes was observed; nevertheless the mitotic figures increased gradually in the germ centers, the height being reached in a few days after the treatment, and the increase persisted for a period of about two weeks.

Lymphoid Reaction in Immunity to Transplanted Cancer Induced by Small Dose of X-Rays in Mice.

Further, it was noted that in mice a certain small dose of x-rays of low penetration induces a stimulation of the lymphoid tissue (Nakahara and Murphy '20) and a regular and considerable increase in the number of mitotic figures of the germ centers takes place from twenty-four hours to four days after the treatment.

When cancer grafts were inoculated in these mice seven days after the x-ray treatment, an appreciable increase in resistance as compared to the normal untreated mice was detected (Nakahara and Murphy, '21). The blood picture of the mice, thus rendered comparatively more resistant, showed no constant change following the x-ray treatment, but the lymphocytes were found to rise 100 per cent or more above the normal within two weeks after cancer-graft inoculation (Nakahara and Murphy, '21). As in other varieties of cancer immunity, the polymorphonuclear leucocytes showed no appreciable change.

There was some variation observed in the histological appearance of the lymphoid organs of mice first x-rayed and inoculated with cancer grafts seven days afterward, but as a rule a profound acceleration of the rate of cell division was present in the lymphoid tissue, and especially in the area of germ centers (Nakahara and Murphy, '21).

DISCUSSION.

The material which supplied the above data was studied primarily from the point of view of our interest in the relationship between the lymphoid cells and resistance to transplanted cancer in mice. In cancer immunity, induced by the usual means, namely, by the injection of living homologous tissue, it was noted that a marked increase in the circulating lymphocytes occurs, with which is associated an increase of cell multiplication by mitosis in the germ centers of the lymphoid organs. This relationship between the resistance to cancer inoculation and lymphocytosis was observed, regardless of means by which the lymphocytosis is induced. The rise of the lymphocytes of the blood is invariably preceded by an increased proliferative cellular activity in the germ centers. These facts suggest that lymphoid germ centers are really made up of lymphoblastic tissue, and are among the sources of lymphocytes. The cells comprising the so-called germ centers and the lymphocytes making up the nodule of lymphoid organs are often stated to differ morphologically, but in reality there is no criterion which serves actually to distinguish one from the other.

The fact that phagocytosis sometimes occurs in the area of germ centers and is absent from the lymph-nodules may be urged as evidence that the cells of the germ center are not lymphoid in nature, as lymphocytes are non-phagocytic. Such a deduction would seem too dogmatic, since embryonic mesenchymal cells, from which lymphoid tissue develops, are admittedly phagocytic. Moreover, it would be difficult to decide whether the large cells containing ingested cell fragments (Flemming's 'stainable particles') really originate from the cells indigenous to germ centers or come from without. It is well known, also, that new lymphocytes are produced by a division of a preexisting one, which is capable of mitosis. This obviously cannot be considered as the only possible method of lymphocytic proliferation, as, embryologically, it is conceivable that cells with the potentiality of developing into lymphocytes may coexist in the adult along with the already differentiated lymphocytes.

SUMMARY.

Several kinds of lymphoid reactions are described in which stimulation of the cell division of the germ centers of lymphoid organs preceded lymphocytosis in the blood. The association of the two processes described provides evidence that the lymphoid germ centers are in fact sources of blood lymphocytes, as the name indicates.

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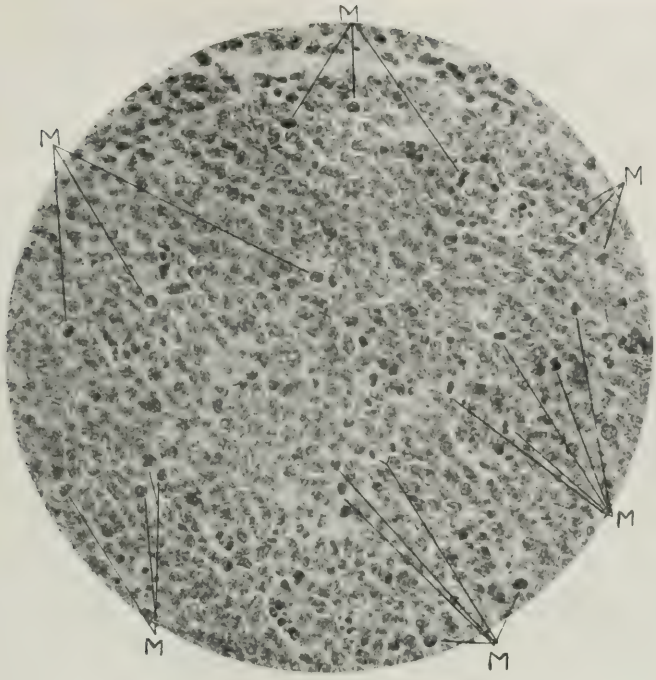
EXPLANATION OF PLATE.

FIG. 1. A germ center of lymph-node of mouse treated with a small dose of x-rays of low penetration. Note the hyperactivity in cell proliferation as indicated by an unusually large number of mitotic figures (*M*). The majority of mice thus treated are potentially more resistant to transplanted cancer than untreated mice, and show, upon cancer inoculation, a marked increase in the number of circulating lymphocytes. × 480.

FIG. 2. An inactive germ center of lymph-node of mouse. Note the absence of mitotic figures. × 480.



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(Nakahara and Murphy: Germ center in lymphoid tissue.)

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STRUCTURE AND SIGNIFICANCE OF THE PHOSPHATIDES.

BY P. A. LEVENE.

BIBLIOGRAPHY.

BY IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The present review is written at the request of the editors of this journal. The author hesitated to accept the invitation, in view of the fact that the literature on the subject has been only recently reviewed in the admirable monograph by Hugh MacLean. The views of this author and of the present writer agree so closely that there seemed little ground for the present undertaking. However, even in the short lapse of time since the appearance of MacLean's work, some new contributions have been made. Furthermore, the present writer was impressed by the argument that it is intended to supplement this review at irregular intervals with further reviews, when the growth of the material of the subject will call for such.[1]

I.

The name phosphatide indicates a substance containing phosphorus in its molecule. There exists, however, a great number of phosphorus-containing organic compounds which are not classified as phosphatides. The name is conventional and is attributed to such esters of phosphoric acid as resemble fats. With these fats they are extracted from animal and plant tissues by the usual fat solvents, and as these, they yield higher aliphatic acid under the action of hydrolytic agents.

In the attempt to classify phosphatides, a great diversity of standards has been employed by individual writers. The older workers and the less critical of the recent workers based their classification either

[1] Since these lines were written two new summaries have appeared, one by Fourneau and the other by Zunz, both very interesting, and both are highly recommended to students on the subject of phosphatides.

entirely on physical properties (Vauquelin, Couerbe, Fremy) or on a consideration of solubility combined with that of the ratios of the nitrogen to phosphorus equivalents in the molecules of individual substances. This mode of classification led to claims for the existence of a great many individual substances, the reality of which today seems doubtful. The discussion of substances of debated reality has no place in a short comprehensive review.

The group of phosphatides whose individuality is based on the study of chemical structure is short in number. It embraces only the three generic substances, lecithin, cephalin and sphingomyelin. MacLean was guided by similar considerations, limiting the discussion in his monograph to four phosphatides: the three just mentioned, and cuorin. Since then, it was shown by Levene and Komatsu that cuorin is a mixture of decomposition products of cephalin and lecithin. This was substantiated by the observation of MacLean who found that strictly fresh heart muscle is free from cuorin. Thus, the number of well defined phosphatides is limited to three. This view was expressed by Levene in 1916 and has recently been accepted by MacLean. The present review will record, therefore, the recent progress of the work on the three named substances.

II. Preparation of Phosphatides.

For the extraction of phosphatides the same solvents in general are used now as were employed by the pioneer workers, namely, alcohol, ether, petrolic ether and acetone. By means of hot alcohol alone all the phosphatides may be extracted. Each of the other solvents is capable of extracting only certain of the phosphatides. Thus, acetone in the presence of fat extracts principally unchanged lecithin and cephalin, the extraction being incomplete. Ether extracts lecithin, cephalin and products oftheir partial hydrolysis, and also small quantities of sphingomyelin.

In practice it was found expedient to select the extracting reagent according to the purpose of the extraction. When it is desired to obtain very pure lecithin or pure cephalin, the acetone extract is employed. When the object of the extraction is the preparation of all unsaturated phosphatides, then ether is chosen as the reagent. Finally, when it is desired to prepare sphingomyelin, the dried tissues are extracted directly by means of alcohol.

As a matter of routine, all three reagents are employed consecutively, the procedure being as follows. The organs are minced and dried in a vacuum drier. The dry material is then pulverized and extracted consecutively with acetone, ether and alcohol. Each of the extracts is worked over separately. Thus three fractions are obtained as follows:

1. The acetone extract contains lecithin and cephalin and only a small proportion of their decomposition products. The fraction of phosphatides obtained by this reagent will be referred to later in the discussion as the acetone fraction. This fraction is the one best suited for the preparation of pure lecithin.

2. The crude etherial extract contains unchanged lecithin and cephalin, associated with the products of their partial decomposition. This fraction can be separated into two subfractions. One is soluble in cold as well as in hot alcohol and is commonly named "lecithin"; and the other is soluble in ether but insoluble in alcohol, and is generally referred to as "cephalin."

3. When desiccated brain powder is extracted with warm alcohol, the clear filtrate on standing gives rise to a white precipitate, the "White Matter" of Vauquelin.

Historical.—The progress of the methods of extraction proceeded as follows. Fourcroy (1793) was practically the first to note the substances which are now referred to as phosphatides. He employed only one extractive, namely, alcohol. Couerbe (1834) then employed both ether and alcohol. Attempts by later workers to replace ether by low boiling gasoline or by benzene have not met with success.

One of the most important advances in the development of the chemistry of phosphatides was made by the introduction of acetone by Altman and Luebser (1899). Acetone was suggested for the separation of unsaturated phosphatides, lecithin and cephalin, from fats and cholesterol. However, Luebser realized that the separation accomplished by it was not complete. Some phosphatides were found in the acetone extract, while the crude acetone precipitate was found to contain a variety of impurities. True, Fränkel and his coworkers claimed to have isolated from the acetone extract peculiar phosphatides differing from lecithin and cephalin. These claims, however, were not substantiated by more recent workers (Levene and

his co-workers). Acetone, nevertheless, remains among the most important reagents in the chemistry of phosphatides.

Other reagents have been introduced principally for the purification of individual phosphatides. Thus, cadmium chloride was first employed by Strecker in 1869 for the isolation of lecithin. This reagent was reintroduced by Bergell in 1900. To Bergell also belongs the credit for simplifying the process of conversion of the cadmium salts of lecithin into the free substances. He suggested for this purpose the use of ammonium carbonate instead of the hydrogen sulfide employed by the older workers. More recently, Levene and Rolf in work as yet unpublished employed for this purpose methyl alcohol saturated with gaseous ammonia.

The methods for the separation and isolation of cephalin have not made as much progress as those of lecithin. Thudichum (1884) advocated the use of lead acetate, and all other workers have separated cephalin from lecithin by means of alcohol.

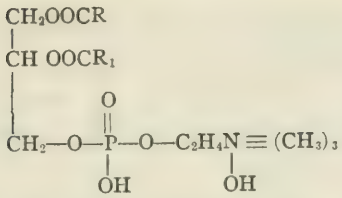
The isolation of the third phosphatide, sphingomyelin, is still very laborious and complicated. Thudichum in 1884 separated it from cerebrosides by fractional precipitation from alcohol, and further purified the substance by means of cadmium chloride. No further advance in the procedure of its preparation was made until 1908, when Rosenheim and Tebb introduced pyridin as reagent. In 1914 a more efficient method was developed by Levene.

In the work on unsaturated phosphatides much use was made in recent years of catalytic hydrogenation. First Paal and later Riedel have shown that lecithin in alcoholic solution can be hydrogenated in the presence of colloidal palladium. Levene and his co-workers have applied the process advantageously for the solution of many problems pertaining to the structure of lecithin and cephalin.

III. Structure of Individual Phosphatides.

In this discussion of the chemical structure of individual phosphatides, first place will be given to the analysis of the present day theory; the older views and the controversial points will then be given in brief in the form of a historical sketch.

A. Lecithin.—The present conception of the structure of lecithin is expressed by the following graphic formula:



(I)

This formula expresses the view that the molecule of lecithin is composed of phosphoric acid combined, on one hand, with a diglyceride, and on the other, with a base, cholin. It further postulates that all parts are linked into one molecule through ester linking. This form of linking lends to the molecule a certain degree of lability.

Components of Lecithin.—Varying the conditions of hydrolysis, it is possible to obtain from lecithin all the components required by theory; namely, fatty acids, glycerol, cholin and phosphoric acid. Naturally only those conclusions as to the nature of the constituents are binding, which have been obtained on material free from impurities. Unfortunately all the older work was done on material which did not meet this requirement. Analyses of pure material were only recently presented by Levene and his co-workers.

Base.—Pure lecithin contains only one base, cholin. MacLean was perhaps the first to prepare lecithin which did not contain amino groups. Levene and Rolf have perfected the method of preparation and have prepared the material in quantities sufficient for complete analysis. The principal impurity of the lecithin of the older writers was cephalin. This was assumed by MacLean and experimentally demonstrated by Levene and West. Hence, material free from aminoethanol may be regarded as lecithin free from impurities, provided its elementary composition agrees with that required by formula (I).

Glycerol.—As regards the presence of this component, the evidence of the older workers was perfectly satisfactory. As early as 1850 Gobley proved its presence beyond doubt, and later Miss Foster found the experimental proportions to agree with those required by the theory. It is true that the material which served for such analyses was not free from cephalin. However, since cephalin and lecithin contain approximately the same proportions of glycerol, the recorded

data on the glycerol estimation are still valid in formulating the theory of the structure of lecithin.

Phosphoric Acid.—The proportion of this component is easily determined from the phosphorus content of the analyzed material. On this point the observations of the older writers are acceptable, since the presence of cephalin and of small proportions of decomposition products should but slightly affect the phosphorus content of a given sample particularly when the limits of error of the method are taken into account.

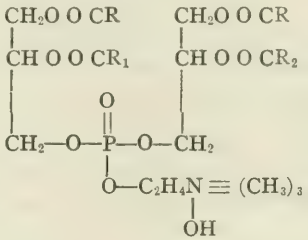
Fatty Acids.—The question of the fatty acids present in the molecule of lecithin deserves special attention. Much of what has been written about lecithins and many of the physiological and biological properties attributed to them were associated with the problem of their fatty acids. The samples of lecithin analyzed in this respect were obtained from egg yolk and from the brain and liver of cattle (Levene and Rolf, Levene and Ingvaldsen, and Levene and Simms). The samples from the egg yolk and from the brain seemed identical in respect to their fatty acids. Both contain one unsaturated and two saturated acids. The unsaturated acid was identified as oleic. In this acid the number of the unsaturated bonds was determined by the iodine and by the hydrogen values. The number of carbon atoms was established by the analysis of the hydrogenated product, which was identified as stearic acid. The saturated fatty acids were found to consist of palmitic and stearic acids. Each of these was isolated in pure state. In every instance the identity of an acid was established on the basis of elementary composition, melting point and molecular weight.

The fatty acids of the liver lecithin differ from those of the lecithins obtained from the other two organs in the character of the unsaturated acids. The latter were found to belong not to the oleic, but to the linolic series, or to a mixture of two acids, one of the oleic and the other of the linolic series. On hydrogenation they were separated into stearic and arachidic acids. The saturated acids of liver lecithin, as of the other organs, are palmitic and stearic acids.

The Bearing of the Findings Regarding the Components of Lecithin on the Theory of Its Structure.—The graphic formula given above admits of only two acyl radicles in the molecule of lecithin. The findings

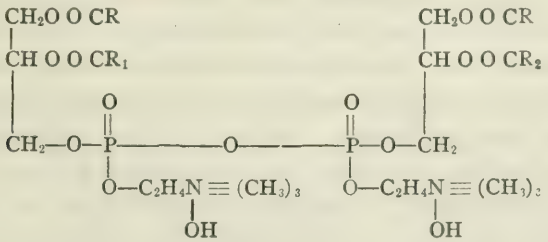
that the combined fatty acids consisted of equal parts of unsaturated and saturated acids, of which the saturated were composed of stearic and palmitic, indicates the presence of four equivalents of fatty acids in the samples of lecithin obtained from egg yolk and from brain. In lecithin obtained from the liver each of the four acyl radicles is distinct in composition.

Assuming that all four equivalents of fatty acids are present in one molecule of lecithin, the graphic formula presented above should be modified to make room for the two additional acyl radicles. A priori, there are possible three alternative arrangements, one in which the remaining free hydroxyl of the phosphoric acid is condensed in an ester linking with a second diglyceride,



(II)

another in which two monolecithins are combined into a dilecithid in the following fashion:



(III)

The third admits of the existence of several monophosphatides each represented by formula (I). The first assumption is excluded unequivocally and with little difficulty on the basis of the elementary composition of lecithin. This is clearly seen from the following table, in which are given the theoretical elementary composition of substances expressed by the formulae (I), (II) and (III), and the analytical data recorded by various observers. It will be seen that formula

(II) is definitely excluded even on the basis of the analytical data of the older writers. The more recent data recorded by Levene and Ingvaldsen and by Levene and Rolf represent a close agreement of the findings with the theory expressed in formula (I). However, it also closely approaches the one of formula (III).

	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Calculated for Formula (I) $C_{44}H_{88}NPO_9$ (M. W. = 805.9)	65.62	11.02	1.74	3.85
Calculated for Formula (II) $C_{81}H_{156}NPO_{13}$ (M .W. = 1382.50)	70.25	11.39	1.01	2.24
Calculated for Formula (III) $C_{86}H_{170}N_2P_2O_{17}$ (M. W. = 1565.89)	65.96	10.93	1.79	3.96
Thudichum (brain)	66.75	10.67	1.81	4.00
Baskoff (liver)	64.64	10.71	1.95	4.00
Stern and Thierfelder (egg)	64.63	10.96	1.79	3.95
MacLean (egg)	64.18	10.60	1.87	3.95
Erlandsen (heart)	66.29	10.17	1.87	3.95
MacLean (heart)	66.27	10.32	1.85	3.97
Eppler (heart)	66.46	10.69	1.87	4.03
Levene and Ingvaldsen (liver)	65.72	10.70	1.97	3.61
Levene and Rolf (egg)	65.56	10.85	2.04	3.87

Still more convincing are the results of the analysis of the hydrolecithin. The physical properties of lecithin are such that one is apt to be skeptical as to the purity of even perfectly pure material. On the other hand, when the substance is hydrogenated it acquires the appearance and the character of a solid wax or paraffin, which is easily purified by recrystallization. Because of this advantage, Levene and his co-workers employed the hydrogenated products for the study of the elementary composition of lecithin and cephalin. The analytical data of hydrolecithin published by Levene and Rolf exclude formula (II), thus leaving under discussion only formulae (I) or (III). Formula (III) does not seem very probable, since a substance of that structure should possess more strongly basic properties than the substance manifests in reality. Molecular weight estimations of hydrolecithin will make possible a final decision between structures (I) and (III). For the present, structure (I) seems in greater accord with the known properties of lecithin.

Accepting formula (I) as the true expression of the structure of lecithin, the finding of four equivalents of fatty acids is most conveniently explained by the third assumption, namely, of the existence of more than one monophosphatide, each of the type expressed by formula (I). On this assumption, egg yolk "lecithin" and brain "lecithin" would both consist of two lecithins, differing in the character of their saturated fatty acid. One might be called stearyl oleyl lecithin, and the other palmityl oleyl lecithin. On the other hand, liver apparently contains two monolecithin phosphatides, each resembling one of those from the egg yolk in the nature of the saturated acid, but both differing from the corresponding substances of the egg yolk by the nature of their unsaturated acids.

On the Configuration of the Lecithin Molecule.—As is obvious from formula (I), the center of the lecithin molecule is occupied by glycerol, a trivalent alcohol $CH_2(OH)CH(OH)CH_2(OH)$ in which every carbon is symmetrical and therefore the molecule is optically inactive.

A derivative of glycerol may be optically active even when all radicles combining with it are optically inactive. The conditions for optical activity in derivatives are presented in the following formulae:

$$CH_2(OR_1)*CH(OR_2)CH_2(OR_3) \quad \text{(IV)}$$

$$CH_2(OR_1)*CH(OR_1)CH_2(OR_2) \quad \text{(V)}$$

The derivative is inactive if the configuration is as follows:

$$CH_2(OR_1)CH(OR_2)CH_2(OR_1) \quad \text{(VI)}$$

When R_2 stands for the cholin ester of a phosphoric acid radicle, and R_2 and R_3 for acyl radicles, then any one of the formulae (IV), (V) or (VI) may express the structure of lecithin. Since every known lecithin contains two different radicles the structure expressed by (VI) probably does not exist in nature. Stearyl oleyl or stearyl linolyl lecithin can exist only in the form having an asymmetric carbon atom. In fact, all samples of lecithin tested for their optical activity have been found dextrorotary. Also, all samples of hydrolecithin have been found dextrorotary. The latter observation is to be expected since palmityl lecithin on hydrogenation still contains two different

* Asymmetric carbon.

acyl radicles. Thus the choice between the two possible formulae (IV) and (V) can not be made on the basis of the properties of the unchanged lecithin.

If instead of lecithin one is dealing with glycerophosphoric acid, the problem is much simplified. While at least three isomers of substituted glycerophosphoric acid are possible, only two stereoisomeric glycerophosphoric acids are conceivable; namely,

$$CH_2(OH)^*CH(OH)CH_2(OR) \qquad \text{(VII)}$$

and

$$CH_2(OH)CH(OR)CH_2(OH) \qquad \text{(VIII)}$$

Of these, formula (VII) expresses the optically active form, while formula (VIII) is optically inactive. Thus, for the solution of the problem of the configuration of lecithin, it becomes essential to isolate the constituent glycerophosphoric acid. It is a matter of good fortune that in the molecule of lecithin the union of glycerol with phosphoric acid is firmer than that with the organic radicles. Owing to this property, it is possible on mild hydrolysis with either alkalies or acids to obtain glycerophosphoric acid. Willstädter and Lüdecke were the first to study the configuration of this acid. They found that it was optically active and hence assigned to the ester, formula (VII) and to lecithin, formula (I). The existence of a lecithin of this configuration is now undisputed. However, in recent years there appears a tendency to accept the existence also of an isomeric lecithin. The structure of this form is assumed to be expressed by formula (IV) in which R_2 stands for the cholin derivative of the phosphoric acid radicle and R_1 and R_3 for two different acyl radicles. The exponents of the new hypothesis are Fourneau and Bailly. The former observed that from the product of alcoholysis of lecithin, glycerophosphoric acid can be isolated as its calcium salt. He succeeded in separating this salt into two fractions differing in their crystalline form. These two salts, he assumed, represented two structural isomers of glycerophosphoric acid. Bailly reached the same conclusion as Fourneau on the basis of the analysis of the products of oxidation of lecithin. He found that one form of glycerophosphoric acid derived from lecithin yielded dioxyacetone while the other failed to do so. On the basis of this observation, he argues that there also exists in lecithin

* Asymmetric carbon.

a glycerophosphoric acid of the structure (VIII), as only this form could yield dioxyacetone.

These two observations of Fourneau and of Bailly are worthy of note and of further investigation. If these conclusions should be corroborated, then the number of isomeric lecithins will be greatly augmented and the entire problem of the structure of lecithin much complicated.

Work of Delezenne and Fourneau on Partial Hydrolysis of Lecithin and Its Significance.—A very important contribution to the chemistry of lecithin was made by Delezenne and Fourneau. They found that the result of the action of cobra venom on lecithin is the formation of a product of partial hydrolysis of the latter. This product is a beautifully crystalline compound containing all the components of palmityl lecithin except oleic acid. This finding is of importance since the presence of four equivalents of fatty acids of which two are of the saturated and two of the unsaturated type, admit of two interpretations regarding the distribution of the fatty acids. One is that each lecithin contains one saturated and one unsaturated acid, and the other admits the existence of lecithins containing only unsaturated, and of types that contain only saturated acids. The fact that the product of Delezenne and Fourneau possesses physical properties different from lecithin and resembling hydrolecithin, points toward the conclusion that if lecithins containing only saturated fatty acid had existed, it should have been possible to isolate them readily from fully unsaturated lecithins. Thus the finding offers additional evidence in favor of formula (I) as expressing the structure of at least one of the isomeric lecithins.

Summary of the Work on the Structure of Lecithin.—The work on the structure of the lecithin molecule may be summarized as follows:

1. It is certain and undisputed that lecithins of the structure expressed by formula (I) do exist.

2. It is practically certain that the monophosphatides differ in the nature of their fatty acids and that hence a variety of different lecithins are possible.

3. It is not certain whether these phosphatides have an independent existence in tissues or exist in form of diphosphatides.

4. It has been suggested, but not yet fully demonstrated, that in addition to lecithin derived from glycerophosphoric acid of formula (VII), there exist also forms derived from the acid of formula (VIII).

Historical.—In view of our present knowledge, it is evident that even the pioneer workers of phosphatides had been dealing with lecithin. The phosphatides which they recognized in the mother liquors from the "white matter" of Vauquelin, could only be mixtures of the phosphatides lecithin and cephalin. It is quite possible that a separation of the two was accomplished by Fremy in 1841. The most fundamental work on lecithin was presented by Gobley. The contribution of Gobley lay in his discovery that glycerophosphoric acid constituted the central part of the molecule of lecithin. He recognized the existence of a base and of fatty acids in the substance. The mode of linkage of the fatty acids and the nature of the base was not explained by this writer.

The nature of the base and the mode of linkage of the fatty acids was established by the work of Diacanow in 1867 and of Strecker in 1868. Between these two writers, however, a controversy arose on the question of the mode of linkage of the base.

Formula (I) expresses the view that the base and the phosphoric acid are linked as an ester through the alcoholic group of the base. The alternative ascribes to the union the character of a complex salt. Diacanow made the latter assumption, while Strecker was the author of the former. Strecker's theory is the one generally accepted today. In recent years this theory was substantiated by the work of Hundeshagen and Gilson.

In this connection, it is interesting to note that Thudichum (1884) always referred to the base having the structure of

$$CH_2 = CHN(CH_3)_3OH$$

as "neurin." This base, by virtue of its structure could be united to the phosphoric acid only in the form of a salt. To some extent the difficulties attached to the work of the older workers on the base of lecithin lay in the fact that they were dealing with samples of lecithin containing varying proportions of cephalin. In recent years MacLean was perhaps the first worker who realized the importance of preparing lecithin free from cephalin. He demonstrated that this end

was attainable through the purification of the cadmium chloride salt. Levene and his co-workers have shown that for the preparation of pure lecithin it is more convenient to start with the acetone extract. The introduction of Van Slyke's method for the estimation of primary amino groups has been of particular service in the preparation of pure lecithin.

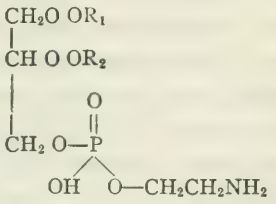
The work on the fatty acids of lecithin up to recent date was in a chaotic state. It was very uneven in character and thoroughness. In a way even the best and most careful of the older work on the fatty acids of lecithin might be dismissed from consideration in the discussion of the structure of lecithin, since all older workers were dealing not with pure lecithin but with a mixture of lecithin and cephalin. On the other hand, the findings of the older writers, particularly if one considers the combined efforts of all workers, are important inasmuch as they have limited the choice of the possible fatty acids in the lecithin molecule. One could scarcely expect to find more acids in pure lecithin than in a mixture of lecithin and cephalin. It must be added, however, that the list of fatty acids isolated from lecithin by the older workers was not complete, since at least one new addition was made by Levene and Ingvaldsen.

The older work on the fatty acids of lecithin is also important for the reason that it has suggested the existence of more than one lecithin. Diacanow, who was the first to publish on the fatty acids of lecithin, characterized the phosphatide as a distearyl derivative; Strecker, immediately after him, isolated oleic acid and two saturated acids, one which analyzed for palmitic (C = 75.07; H = 12.60; M. P. = 56.7°) and to which he refers as margaric, and the other analyzing for stearic acid. Diacanow replied with a statement accepting the existence of a lecithin having the structure of that described by Strecker, but at the same time insisting on the existence of the distearyl lecithin. Later Thudichum criticised Diacanow's theory of the distearyl form. This criticism still holds. The most careful of the modern work on the fatty acids, particularly on the question of the unsaturated acids, is that of Cousin. He isolated two acids, oleic and linolic, from the mixture. The latter was, in the light of present knowledge, derived from cephalin. In chronological order, the authors who contributed to this part of the problem of lecithin structure are as

follows: Diacanow (1867), Strecker (1868), Thudichum (1884), Ulpiani (1901), W. Koch (1902), Cousin (1903 and 1906), Dezani (1909), Serano and Palozzi (1911), Delezenne and Fourneau (1912), Paal and Oehme (1913) and Ritter (1914).

In connection with the problem of the fatty acids, a certain importance is attached to the work on the iodine value of lecithin. Since the unsaturated acid is the only portion of the molecule capable of absorbing iodine, the iodine value of the lecithin may serve on the one hand to determine the degree of unsaturation of the acid, and on the other to establish the ratio of the amount of unsaturated to saturated acids. The combined results of all the work in this field tend to show that "lecithins" of different origin differ in the degree of unsaturation. Since all the writers dealt with impure lecithin the conclusions are not convincing, but on the other hand, in the light of recent work on liver lecithin the findings of the older workers may serve as a guide to future investigators. The most exhaustive study in this direction is that of Cruickshank in 1914. In chronological order the contributors to this problem are Stern and Thierfelder (1907), Erlandsen (1907), Baskoff (1909), Rollet (1909) and Cruickshank (1914).

B. Cephalin.—It is quite certain that cephalin is closely related to lecithin. Its nucleus, as that of lecithin, is a glycerophosphoric acid. As in lecithin, this is conjugated with two fatty acid radicles and with one base. Granting that these statements are correct, the structure of the molecule of cephalin may be formulated as follows:



(IX)

Although there is every reason to believe that this formula is the true expression of the structure of cephalin, yet it is difficult to substantiate it by the analytical data obtained on the material which the workers beginning with Thudichum named "cephalin".

From the standpoint of the character of individual components the experimental findings were entirely consistent with the requirements of the theory. The difficulty arose when one compared the actual elementary composition of the phosphatide with that required by the theory. For many years this difficulty could not be overcome. However, it will be seen from the following discussion that some of the newer work on the subject has brought forth facts which satisfactorily explain the discrepancy between the analytical data of cephalin of the formula (IX) and of that actually prepared in course of the older work.

Components of Cephalin: Phosphoric Acid and Glycerol.—The presence of phosphoric acid in the molecule is demonstrated not only by the result of complete hydrolysis of cephalin which yields phosphoric acid as one of the products, but also by the results of partial hydrolysis, which leads to the formation of glycerophosphoric acid (Fränkel and Dimitz, Levene and Rolf). The question of the presence of glycerol in the molecule of cephalin was satisfactorily proven quantitatively as well as qualitatively. The proportion of glycerol found in the analyzed samples approached satisfactorily that required by the theory expressed in formula (IX), (Foster, Levene and West, Levene and Komatsu).

Base.—The nature and the number of bases present in cephalin has been a matter of considerable discussion in the past. The present evidence suggests the view that a single base is present in the molecule of cephalin, and that this is aminoethanol (Baumann and Renall). This base has been identified among the products of hydrolysis through its gold salt. The assumption that aminoethanol is the sole base of the cephalin molecule is founded on the fact that it is possible to prepare samples of cephalin in which all nitrogen is present in form of primary aminogroups. Since all other bases which different authors claimed to have isolated from the lecithin-cephalin fraction were methylated derivatives of aminoethanol, it is evident that a fraction containing all of its nitrogen in form of primary amino groups can contain only the one base aminoethanol.

Fatty Acids.—The problem of the nature of the fatty acids of cephalin has passed through the same phases of discussion as the question of the fatty acids of lecithin. The present day evidence points toward the existence of one unsaturated acid linolic (Parnas) and one saturated

acid, stearic (Parnas, Levene and West). One must bear in mind, however, that to many workers as well as to the present writer, it seemed for a long time as if the molecule of lecithin contained one saturated acid. Later by means of fractional distillation of the esters it was possible to isolate two saturated acids from the products of hydrolysis. This method of analysis has not yet been applied to the study of cephalin. Likewise, the problem of the unsaturated acids cannot be considered as definitely closed.

The Components of Cephalin and Their Bearing on the Theory of its Structure.—The components of cephalin thus far identified among the products of its hydrolysis are phosphoric acid, glycerol, aminoethanol, stearic and linolic acids. Assuming that all components enter the molecule of lecithin in equivalent proportion, one readily arrives at the structure expressed in formula (IX). This theory requires for cephalin a certain elementary composition and a definite proportion of the component parts. Neither one of these requirements was satisfied by the analysis of the samples of cephalin in possession of the earlier workers.

Thus the elementary composition required by theory is:

	C	H	N	P
Per cent	66.16	10.57	1.88	4.18

whereas the samples of the material used for the different investigations on the nature of the components had varying elementary composition, as given in the following table.

AUTHOR	SOURCE	C	H	N	P	NH_2	Me
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Thudichum	Brain	60.00	9.38	1.68	4.27		
Zuelzer	Ox brain	60.20	9.80	3.80	2.60		
Koch	Sheep brain	59.50	9.80	1.75	3.85		1.73
Cousin	Brain			1.82	3.89		
Stern and Thierfelder	Egg yolk	59.68	9.74	1.57	3.64		
Falk	Nerves	55.75	9.66	1.94	4.42		
	Human brain	57.56	9.21	2.93	3.23		
Neubauer	Brain	61.99	9.85	1.65	3.44		
		62.12	9.87	1.69	3.45		

AUTHOR	SOURCE	C	H	N	P	NH_2	Me
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Parnas	Brain			1.83	3.86		
Frank	Liver	57.10	9.62	1.72	3.91		
Baumann	Human brain			1.84		1.63	
Renall	Ox brain			1.69	3.56	1.37	
	Sheep brain			1.92	4.27	1.60	
Bürger and Beumer	Erythrocytes			1.78	4.06		
Levene and West	Brain	60.49	9.52	1.96	3.80		
Levene and West	Egg yolk	60.00	9.62	1.78	3.69		
Levene and West	Liver	60.33	9.01	1.86	3.75		
Levene and West	Kidney	60.17	8.95	1.70	3.65		

It is evident that the elementary composition recorded in the above table contradicts the theory expressed in formula (IX).

The results of the quantitative analysis of components were found by Levene and West (Cephalin II) as follows:

	CALCULATED	FOUND
	per cent	*per cent*
Base	8.2	8.26
Glycerol	12.4	10.00
Phosphoric acid	13.2	12.17
Fatty acids	75.9	63.40

Thus again in this respect there is lack of agreement between the theoretical requirement and the experimental data.

The problem remained in this puzzling state until the work of Levene and West on the so-called hydrolecithin. From this material a fraction was isolated which contained all its nitrogen in form of primary amino groups which on hydrolysis yielded aminoethanol and which had an elementary composition approaching the one required by formula (IX). Similar material, though not so pure, was obtained by Levene and Komatsu from the heart muscle, and by Levene and Ingvaldsen from the liver.

	C	H	N	P	$\frac{NH_2N}{\text{Total N}}$
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Required	65.81	11.05	1.87	4.15	100.0
Levene and West, found	65.33	10.50	1.94	3.87	98.3
Levene and Komatsu, found	65.50	10.36	1.80	4.29	77.0
Levene and Ingvaldsen, found	66.05	10.62	1.66	3.87	80.7

Later, Levene and Ingvaldsen obtained a sample of unsaturated mixed lecithin and cephalin, in which cephalin was predominating, and which had an elementary composition approaching that required by formula (I), or (IX); namely,

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Per cent	66.00	9.5	1.49	3.60	66.4

Similar material was obtained by Levene and Rolf from the brain of cattle; namely,

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Per cent	65.80	10.37	1.41	3.77	84.00

A comparison of the older and newer findings regarding the substances discussed in this section leads to two alternative conclusions: either there exist two phosphatides in which all the nitrogen is present in form of aminoethanol, or there exists only one cephalin having the elementary composition required by formula (IX), and the "cephalin" of the older writers is a partially decomposed true cephalin. The latter of the two conceptions is the simpler. It is substantiated by the fact that so-called "cephalin" contains all the components required by formula (IX). Furthermore, the quantitative estimation of the components indicates that such material shows a deficiency in the fatty acid content in comparison with the theoretical. Such a deficiency may well account for the low carbon content of the old type cephalin. For the final decision, it will be necessary to prepare sufficient material having the composition required by formula (IX), and to demonstrate that it contains the same fatty acids as those isolated from the "cephalin" of the older writers.

Recent work on cuorin by Levene and Komatsu and by MacLean, and the work on heparphosphatide by Levene and Ingvaldsen, bring forth additional facts which help to elucidate the relationship of the old type cephalin to the substance of the composition expressed by formula (IX).

Cuorin and Heparphosphatide.—Cuorin was regarded as an independent individual phosphatide. Levene and Komatsu have shown that it was composed of a variety of fractions. One of these had the composition of the so-called cephalin. This material could then be further fractionated and one of the fractions had the elementary composition required by the theory for definite intermediate products of degradation of cephalin. One of them very closely resembled the monostearyl glycerophosphoric ester of aminoethanol, for which

	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
The theory requires	57.49	9.97	2.91	6.45
And analysis found	56.93	9.23	2.14	6.05

On the other hand, MacLean has shown that when the organs used for preparation of cuorin are fresh, the material obtained in place of cuorin has the composition of the so-called cephalin. Thus these findings point towards the theory that so-called cephalin is an intermediary phase between true cephalin and cuorin, the latter being deficient not only in fatty acids but also in base.

All that has been said in regard to cuorin may be repeated in the discussion of the heparphosphatide of Baskoff. Levene and Ingvaldsen have shown that from comparatively fresh organs, instead of heparphosphatide, a material is obtained having the composition of cephalin, and again this substance was shown to consist of a variety of fragments.

In summing up all the work on the correlation of elementary composition to the chemical structure of the phosphatides containing aminoethanol as base, it may be assumed that there exists in all tissues, first, a true cephalin having the composition required by formula (IX), and second, in tissues in a state of imperfect freshness, substances are found which differ in their elementary composition from formula (IX), but contain all the components required by it. To this group of substances belongs the "cephalin" of the older writers, together with cuorin and heparphosphatide. Whether their formation is entirely a post-mortem process or partly intravital, remains to be established. In part undoubtedly the process is post-mortem.

Configuration, Possible Isomerisms and Further Peculiarities of the Cephalin Molecule.—The problem of the configuration of cephalin, as that of lecithin, pivots on the possibilities in the variation of the grouping of phosphoric acid and of the two organic acid radicles on the glycerol. If our present information on the fatty acids of cephalin is correct then the problem of configuration of this phosphatide is simpler than that of lecithin. The reason is the following. Since linolic acid is hydrogenated into stearic acid, the hydrogenated cephalin is to be regarded as a distearyl phosphatide and may have one of the two alternative configurations as follows:

$$CH_2(OR_1)CH(OR_2)CH_2(OR_1) \qquad \text{(the same as VI)}$$

or

$$\underset{(1)}{CH_2(OR_1)}{}^{*}\underset{(2)}{CH(OR_1)}\underset{(3)}{CH_2(OR_2)} \qquad \text{(X)}$$

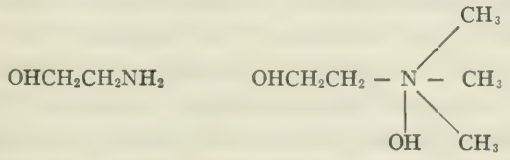
(In these, R_1 stands for a stearyl and R_2 for a phosphoric acid radicle.) Only formula (X) contains an asymmetric carbon, and since hydrogenated cephalin was found optically active by Levene and West, the structure (X) should be accepted. This theory is further substantiated by the fact that Levene and Rolf have isolated a levorotary glycerophosphoric acid from a sample of cephalin which was free from any traces of lecithin. True, in unreduced cephalin there is possible a structural isomerism depending on the respective positions of linolic and stearic acid radicles in the positions (1) or (2) of formula (X). At present there are no data permitting a choice between these two possibilities.

In connection also with cephalin one must bear in mind the possibility of the co-existence of two isomers derived from the two isomeric glycerophosphoric acids, as is claimed by Fourneau and his co-workers, and by Bailly, for lecithins. Fortunately for the student, no observations justifying such an assumption have as yet been made.

There remains to be discussed one peculiarity of cephalin which has been the cause of much speculation. While the hydrolysis of lecithin by means of acids or alkalies proceeds comparatively smoothly so that one finds no difficulty in isolating nitrogen-free glycerophosphoric acid and a nitrogen-free fatty acid fraction, the hydrolysis of cephalin seems to proceed at a much slower rate, so that prior to the work

of Levene and Rolf it was found difficult to prepare from cephalin a glycerophosphoric acid free from nitrogen. Furthermore, the fatty acid fraction was often contaminated with phosphorus containing fragments. To the mind of many workers this peculiarity in the behavior of cephalin militated against the theory that it was a derivative of glycerophosphoric acid in the same sense as lecithin. This skepticism, however, is not warranted. Levene and Yamagawa, in their work on phosphoric acid esters of substituted glucosides, have shown that the rate of hydrolysis of phosphoric acid in those compounds is influenced by the nature of the substituents of the sugar molecule. On the basis of their observations, it is possible to conceive that the differences in the rate of hydrolysis of the phosphoric acid in lecithin and cephalin may be caused by differences in the nature either of their respective bases or of the fatty acid radicles. Further work in this connection is still needed.

Relation of Lecithin to Cephalin.—The final conclusion regarding the respective structure of lecithin and cephalin is that they differ unequivocally in the nature of their bases, one containing aminoethanol, and the other its methylated derivative, cholin.



aminoethanol cholin

As regards the fatty acid, cephalin differs from lecithin as much as various lecithins differ among themselves.

Historical.—The history of cephalin is less complex than that of lecithin. In a way it is more satisfactory since nearly every new publication on the subject marked a real progress, large or small, in the intimate knowledge of this phosphatide. Fremy in 1884 described an ether-soluble phosphatide which was insoluble in alcohol. In the light of subsequent knowledge he was dealing with cephalin. Thudichum (1884) was the first to individualize cephalin as a phosphatide distinct from lecithin. The differences according to Thudichum consisted in the nature of the fatty acids and of the bases.

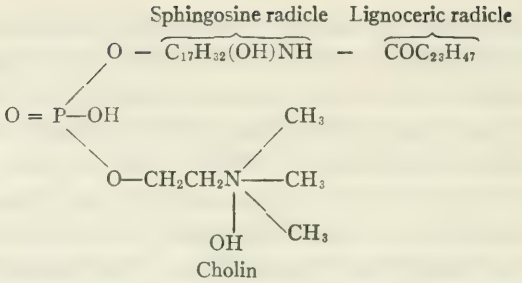
Whereas the unsaturated fatty acid of lecithin was identified as oleic, he described that of cephalin as cephalinic acid. He was unable to assign a structure to this acid, but he attributed the peculiarities of cephalin to this radicle. The basic radicles discovered by Thudichum in cephalin were neurin (cholin), aminoethanol, and a third base, not identified. However, in discussing the possible structure of cephalin he takes account only of cholin on the assumption that the other bases were degradation products of it. Thudichum was also the only writer who emphasized the discrepancy between the empirical elementary composition of cephalin and the one required for it by theory. The part played by Thudichum in the development of the theory of the structure of cephalin is typical of all his work on the chemistry of the brain. His general conceptions were usually correct. He had a wonderful chemical instinct for sensing distinction when such had been overlooked. He applied better chemical methods than his predecessors and many who followed him, but he nearly always failed in the details. In fact he often erred so much in this respect that entire chemical structures formulated by him had to be recast so as to make the true details fit the whole. So in connection with cephalin, the true structure of the components was developed by a succession of subsequent workers. The true nature of cephalic acid has been recognized as linolic acid. Cousin was the first to suggest this hypothesis on the basis of the iodine value of the substance; Falk supported the same view on the ground of the hydroxy derivatives obtained from it. Finally, Parnas reached the same conclusion working on the hydrogen absorption of the acid and on the product of its hydrogenation. Thus there gradually accumulated an abundance of evidence supporting the view that the cephalinic acid of Thudichum was in reality linolic acid. In 1916 there appeared a publication by MacArthur, which places the question in an entirely different light. With Thudichum, MacArthur accepts the existence of a true cephalinic acid of the composition $C_{16}H_{32}O_3$, but he also accepts the existence of two other acids, oleic acid and clupandonic ($C_{18}H_{28}O_3$) or an acid $C_{20}H_{32}O_2$. He, however, admits that the evidence submitted by him is not conclusive. Which of the two views on the nature of the unsaturated acid will survive remains to be seen from future experiments.

The ideas on the basic component of cephalin have likewise crystallized gradually. The original work of Thudichum was followed by that of Koch (1902) in which the suggestion was made that the base was of the nature of a monomethylamine. The same conclusion was reached by Fränkel and Neubauer in 1909. On the other hand, Cousin in 1907 could isolate from cephalin no other base than cholin. In the light of subsequent work it is evident that these three workers were dealing with material mixed with lecithin. The state of knowledge of the structure of the base for a time remained stationary until a new impetus was given to the work by the contribution of Trier who isolated aminoethanol from plant phosphatides. Soon Baumann (1913) and Renall (1913), working under the direction of Parnas, identified this base among the products of hydrolysis of cephalin. Finally, the application of the Van Slyke method of estimation of the unsubstituted amino groups brought about a further progress in the work on cephalin. It was shown that this phosphatide contained all its nitrogen in form of primary amino groups. True, the samples of cephalin analyzed by Baumann and by Renall were not yet absolutely pure. Even the purest samples contained a few per cent of non-amino nitrogen, some contained more than 10 per cent. The proportion, however, was comparatively small and also varying, so that it justified the conclusion that amino-ethanol was the only base present in cephalin, since no other bases with a primary amino group have been found among the components of phosphatides.

C. Sphingomyelin.—Sphingomyelin is a phosphatide which differs in its physical and chemical properties from both lecithin and cephalin. On the basis of the most recent work, its chemical structure can be expressed by the following graphic representation.

The Components.—Formula (XI) assumes that the molecule contains four components in equivalent proportion; namely, phosphoric acid, an organic acid, and two bases, sphingosine and cholin.

Phosphoric acid is readily liberated from the molecule on hydrolysis with either acids or alkalies.



(XI)

Bases.—Sphingomyelin is the only phosphatide which has been definitely shown to contain two bases in its molecule. It is the only one that may be correctly named a diamido-monophosphatide. In this sense the other two phosphatides, lecithin and cephalin, are monoamido-monophosphatides. Since this manner of characterization in this instance proved to be correct and serviceable, the majority of workers on lipoids have extended its use to the characterization of many substances of doubtful existence. There have been described monoamido-diphosphatides, triamido-diphosphatides, and a variety of other combinations. For the present most of the claims for the existence of such substances are not justified.

Cholin.—$OHCH_2CH_2N(CH_3)_3OH$ has already been mentioned in the section on lecithin. Its cohesion with the rest of the molecule of sphingomyelin seems to differ from that of the other organic radicles. Thus, by barium hydroxide hydrolysis cholin is completely detached at a time when the other components apparently still remain united. It may be isolated in the form of its picrate and identified as the salt of chlorplatinic acid.

Sphingosine.—This in many ways is a unique substance. As yet it has not been discovered in plants. While cholin and aminoethanol are known to exist also as components of plant phosphatides, sphingomyelin and sphingosine have thus far been isolated only from animal tissues.

The graphic presentation of the base, as far as present knowledge permits it to be formulated, is as follows:

$$\underset{5}{CH_3(CH_2)_{11}}\underset{4}{CH} = \underset{3}{CH}\underset{2}{CH(OH)}\underset{1}{CH(OH)CH_2NH_2}^{*} \qquad \text{(XII)}$$

* The author takes this occasion to express his appreciation to H. MacLean for calling his attention to an error in the article on sphingomyelin. Referring to dihydrosphingosine, the present author gave the correct graphic formula, but in the text the number of hydrogen atoms is given as 35 instead of the correct number of 33.

This formula represents the substance as a primary amine containing seventeen carbon atoms united in a normal chain. Thudichum determined its elementary composition and Levene and West have established the structure of the carbon chain as containing one unsaturated bond. Levene and Jacobs were the first to demonstrate the latter point by converting the base into its dihydro derivative. Sphingosine contains, besides the primary amino group, two hydroxyls, as was demonstrated by the formation of a triacetyl derivative. The latter was prepared independently by Levene and Jacobs and by Thierfelder and Thomas. These groups give to the base an important position in the structure of the phosphatide.

The further details of the structure of the base were worked out by Lapworth and by Levene and West. The first of these writers has shown that on oxidation with chromium trioxide the base yielded a tridecylic acid. Through this finding he established the fact that the carbon atom carrying a double bond and the three substituted carbon atoms constituted a chain of four, attached to the other chain of thirteen. Levene and West have shown that the double bond was situated in the position 4, 5 and that hence the three substituted carbon atoms constituted an uninterrupted chain. In an unpublished work, Levene has converted the base into the normal heptadecan. The respective allocation of the amino and hydroxyls is not yet established, nor is the configuration of the two asymmetric carbon atoms. Levene and West made an attempt to determine the latter but reached no convincing conclusion. The significance of the knowledge of the structure of the base to that of the phosphatide will be discussed later.

Fatty acids.—Two fatty acids have been isolated from sphingomyelin. The composition of the mixed fatty acids was found by Levene as follows:

	LOWEST	HIGHEST	REQUIRED FOR STEARIC ACID
	per cent	*per cent*	*per cent*
C	75.49	76.75	75.98
H	12.71	13.00	12.76

Apparently on the basis of similar findings, Thudichum assumed the acid to be an isomer of stearic, since it melted at 57°C. whereas stearic melts at 70 to 71°C. By fractional distillation of the methylesters of the mixed acids, it was possible to separate them into two fractions. One consisted of the methylester of lignoceric acid and the second fraction analyzed for the methylester of a fatty acid with a lower carbon content. The true nature of the second is not well established, although it seemed to analyze for hydroxy stearic acid. The proportions of the two acids seemed to be equimolecular (Levene).

The Structure of the Sphingomyelin Molecule.—Formula (XI) represents the molecule of sphingomyelin as consisting of four components in equivalent proportions. Since two fatty acids have been isolated from sphingomyelin, one is again confronted with two alternative possibilities; either the substance is a diphosphatide, or a mixture of two individual phosphatides. For the present there exist no data permitting a choice between the two views. There is, however, sufficient evidence to support the theory that each monophosphatide is composed in the manner expressed by formula (XI). The supporting data are found in the results of elementary analysis and in those of quantitative hydrolysis. The results are given in the following table:

	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Lignoceric phosphatide (calculated)	67.48	11.61	3.42	3.79
Oxystearyl phosphatide (calculated)	64.89	11.21	3.78	4.19
Equivalent mixture of the two (calculated)	66.24	11.49	3.59	3.97
Sphingomyelin (Levene) (found)	66.59	11.34	3.66	3.98
	FATTY ACIDS		SPHINGOSINE	
	per cent		*per cent*	
Theory for the equivalent mixture	43.00		35.29	
Sphingomyelin (Levene) (found)	43.00		34.10	

The nitrogen content of the crude base was found 4.5 per cent whereas the theory requires 4.91 per cent.

Thus that part of the theory that assumes in sphingomyelin-mono-phosphatide a union of the four enumerated components is

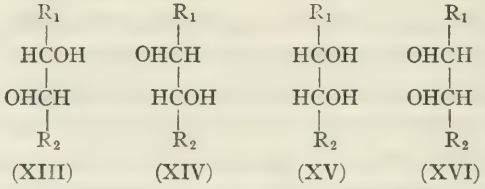
definitely established. The part of the theory expressed in formula (XI) which deals with the mode of linkage of the four components is, in certain points, supported by experimental evidence; in the main, however, it is based on theoretical considerations.

As in lecithin the glycerol molecule may be regarded as the bridge connecting the phosphoric acid and the acyl radicles, so in sphingomyelin the central rôle can be assigned only to the sphingosine which carries two hydroxyls and amino groups serviceable for union with other radicles. Thus in truth in sphingomyelin one of the hydroxyls of sphingosine is united in ester form with the phosphoric acid radicle and the amino group is united with the acyl radicle. This latter assumption is based on experimental evidence. First, sphingomyelin contains no free primary groups in its molecule; and second, on hydrolysis of sphingosine, an intermediary substance was isolated which analyzed for lignoceryl sphingosine and which also contained no free primary amino group.

Accepting then the positions of the acyl and phosphoric acid radicles as given in formula (XI), there remain but two alternative possibilities for the allocation of the base cholin. A priori, it might combine either with the phosphoric acid in ester form as represented in formula (XI) or to one of the hydroxyls of sphingosine in ether form. Since it was found that on hydrolysis of sphingosine cholin is detached very readily, and since a loose union is characteristic rather of an ester than of an ether linking, the preference is given to formula (XI). Furthermore, if the base were linked to the sphingosine molecule the phosphoric acid radicle in sphingomyelin should function as a monosubstituted phosphoric acid and should lend to the phosphatide well-defined acidic properties. In another point, formula (XI) is entirely arbitrary. As seen from formula (XII), the phosphoric acid radicle may be attached equally well in the position 2 or 3. At present there are no data permitting a choice between the two possibilities. Furthermore, one must remember that in formula (XII) the allocation of the primary amino group is as yet arbitrary. It may be allocated as well in position 2 as in 3, thus the positions of the hydroxyls might accordingly be different.

Also, one must remember that regardless of the respective positions of the hydroxyls and of the primary amino group, the sphingosine

molecule permits of four possible stereoisomers as presented in the following.



In these graphic presentations, R_1 stands for CH_2NH_2 and R_2 for the rest of the molecule (carbon atoms 4 to 17).

A priori, it is not impossible that more than one isomer of the base exists in nature, and because of this, theoretically one must admit the possibility of the existence of several stereoisomeric sphingomyelins. On the other hand, accepting the existence in nature of only one form of sphingomyelin, one is not as yet in a position to assign its true configuration.

Occurrence of Sphingomyelin in Different Organs.—Like lecithin and cephalin, sphingomyelin undoubtedly is a common constituent of cellular material of the animal kingdom. It has been isolated from all those organs in which it has been sought. In regard to both composition and physical properties, samples of different origin seem to show little variation. The following table represents the results of the analyses of some samples from various organs.

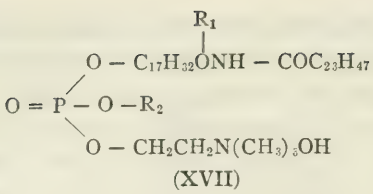
	C	H	N	P	$[\alpha]_D$	SPHINGOSINE	ACIDS
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*
Brain	66.59	11.34	3.66	3.98	+8.20	34.10	43.00
Kidney	64.80	11.41	3.50	3.82	+8.73	32.10	49.00
Liver	64.47	11.57	3.41	3.81	+7.61	32.14	41.70
Egg yolk	65.56	11.68	3.84	4.22	+7.54	33.70	43.40

Physical Properties of Sphingomyelin.—Sphingomyelin crystallizes in very thin plates, often congregating in form of rosettes. Dried, it has the character of a white powder of slightly waxy character.

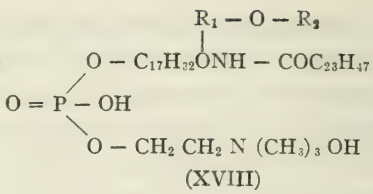
Test of Purity.—The orcin test with or without copper may be used as a test of purity of sphingomyelin. Pure sphingomyelin contains no galactose in its molecule; on the other hand, the principal impurities of the phosphatide are the galactosides.

Historical.—The name sphingomyelin was first introduced by Thudichum. In the light of modern investigation, the material in his hands was a mixture of sphingomyelin with a small proportion of cerebrosides. Since cerebrosides and sphingomyelin are soluble in hot alcohol and insoluble in cold, they are simultaneously extracted, when hot alcohol is used as the extractive. They are also simultaneously deposited as a white precipitate on cooling the hot extract. In this manner this mixture was first obtained by Vauquelin in 1812 and described as the "substance blanche." Couerbe in 1834 described it as "cerebrot," and Fremy in 1841 as "cerebric acid." In 1865, Liebreich prepared from this material a substance named by him protagon, and soon afterwards there began an intermittent controversy as to the real existence of this substance. Diacanow and Hoppe-Seyler were the first opponents to the Liebreich theory. Thudichum, who isolated his sphingomyelin from the so-called protagon, saw more clearly than the other workers the true nature of protagon. However, the controversy continued to our day, when it was conducted by Gies and his co-workers in the negative and by Cramer in the affirmative. A very complete historical sketch of the controversy is given by MacLean in his monograph of *Lecithin and Allied Substances.*

It must be said that the tenacity of the controversy was due principally to the lack of information on the chemical structure of sphingomyelin. On the basis of the elementary composition, protagon can be conceived as consisting of one equivalent of sphingomyelin and two of cerebrosides. From formula (XI), it is evident that there are two positions which could readily serve for linkage of cerebrosides to the phosphatide; namely, the two free hydroxyls. Cerebrosides also can unite with other groups most readily through their hydroxyls. Thus the union of a cerebroside to the sphingosine of the sphingomyelin would result in an ether linking. Such a linking is characterized by its great stability. The second cerebroside radicle could then be united either to the phosphoric acid in an ester linking, or as a dicerebroside in which the cerebrosides are united between themselves in ether form. Thus protagon should have had one of the two following structures:



(XVII)



(XVIII)

There is no reason to believe that substance (XVII) or (XVIII) should be so unstable as protagon was found to be.

The most important work of recent years which helped to clarify the confusion of ideas on protagon was that of Rosenheim and Tebb in 1908. These authors introduced pyridin as a reagent for the separation of sphingomyelin from cerebrosides. The product obtained by them must be regarded, in the light of subsequent work, as crude sphingomyelin. As regards the theory of the structure of the substance, Rosenheim and Tebb were ready to accept that advanced by Thudichum. In the evolution of the theory of the structure of sphingomyelin the merit of Thudichum consisted in recognizing the fact that sphingomyelin did not contain glycerol, but that it contained two bases, cholin and sphingosine. However, he isolated the bases only in a state of great impurity, and hence the presence of sphingosine in the molecule of sphingomyelin was made probable rather than proven. Furthermore, Thudichum accepted the existence in the phosphatide of a higher alcohol, sphingol. In formula (XI), as advanced by Levene, there is no room for that alcohol. The nature of the fatty acids was not recognized by Thudichum. The theory of the structure developed in this review is based principally on the work of Levene published in 1916. The following table, taken in the main from the monograph of MacLean, records practically all the occasions in which individual workers dealt with crude sphingomyelin.

D. Physico-Chemical Properties.—The physico-chemical properties of all phosphatides may be discussed in one chapter for the reason that in this respect they are very similar, the possible differences being only of quantitative nature.

As seen from their structural formulae, all possess amphoteric properties. In the free state they are only moderately soluble in water and are non-diffusible through such membranes as are impermeable to proteins. Thus from the viewpoint of their physico-chemical prop-

erties, phosphatides resemble proteins and hence it is logical to expect the action of electrolytes on each of the two classes of substances to be similar.

	C	H	N	P	
	per cent	*per cent*	*per cent*	*per cent*	
Couerbe (cerebrot) 1834	67.82	11.10	3.40	2.33	Brain
Thudichum (sphingomyelin) 1884	65.37	11.29	2.96	3.24	Brain
Thudichum (apomyelin) 1884	67.10	11.35	3.00	3.23	Brain
Thudichum (amidomyelin) 1884	62.4				Brain
Stern and Thierfelder (Weisse Substanz) 1907	68.15	12.14	2.77	3.22	Egg-yolk
Rosenheim and Tebb (sphingomyelin) 1908	62.90	11.54	3.3	3.46	Brain
Dunham and Jacobson (Carnaubon) 1910.	67.12	11.54	2.84	2.18	Ox kidney
MacLean 1912	68.19	12.37	3.00	3.44	Horse kidney
MacLean 1913			3.07	3.41	Ox heart

Taking into account the results of the recent work on proteins by Jacques Loeb, one may expect that the interaction between phosphatides and electrolytes should be determined by the hydrogen ion concentration of the surrounding medium. Speaking in terms of pH, at a pH value higher than that of their isoelectric point, the phosphatides should act as anions and conversely at a lower pH value as cations.

On the basis of the work of Jacques Loeb on proteins, it again should be expected that the osmotic pressure of an aqueous emulsion of lecithin or other phosphatides would be influenced by electrolytes in the sense of the Donnan membrane equilibrium. Unfortunately all the work on the action of electrolytes on phosphatides antedates the recent work of Loeb and is based on the older conceptions of colloidal chemistry. But such experimental data as exist might be employed in support of the newer viewpoint. The intention of the individual workers was to establish a complete parallelism between the nature of salt action on proteins and on phosphatides. Admitting then that this parallelism has been established and admitting further that the true chemical mechanism of the interaction between electrolytes and proteins has been clarified by the work of Loeb, one naturally feels justified in applying the reasoning of Loeb for the interpretation of the mechanism of the interaction between electrolytes and phosphatides.

These introductory remarks, it is hoped, will facilitate the appreciation of the true value of the older work. In 1868, Strecker formulated the structure of salts of lecithin with neutral inorganic salts analogous to the salt of sodium chloride and glycocol.

$$\begin{array}{l} CH_2NH_2\,HCl \\ | \\ COONa \end{array}$$

The cadmium chloride derivative and the chlorplatinate of lecithin had according to Strecker a structure analogous to that of complex salts.

More recent work dealt principally with the precipitation phenomenon. Waldemar Koch, and Pages and Neubauer, were the principal authors who contributed to this subject. They explained the phenomenon in the light of the views of colloidal chemistry which then came to the foreground. Pages and Neubauer were particularly emphatic in claiming a complete analogy between the action of salts on phosphatides and on proteins. Thus, in their observations the action of anions seemingly followed the Hofmeister series, and the precipitation series seemed to them analogous to those observed by Pauli for proteins. In the precipitation effect of $FeCl_3$ on lecithin Pages and Neubauer saw an action of two colloids of unlike electric charge, while the precipitation of lecithin by cadmium chloride was interpreted by them as an adsorption phenomenon. Similar to the older workers on proteins, these were ignorant of the influence of the concentration of the hydrogen ions on the combining power of amphoteric substances, and like those older workers on proteins, they came to erroneous conclusions.

The work of Thomas in 1915 is particularly interesting. He found that the addition of electrolytes brings about a fall in the osmotic pressure of a lecithin emulsion. An identical effect had been previously observed in the action of electrolytes on proteins (Loeb, Lillie, Pauli and others). In connection with proteins the phenomenon has been explained by Loeb on the basis of "membrane action" discovered by Donnan in 1911. Donnan's law undoubtedly explains also the observations of Thomas.

Thus it is obvious that a revision of the older work on the physicochemical properties is much needed in order to fortify, by experi-

mental proof, conceptions which a priori seem very probable. In connection with such work, one may mention that of Feinschmidt done in the laboratory of Michaelis. This author made an attempt to establish the isoelectric point of lecithin and found that it varied for different samples, occurring at a pH value varying between 2 and 4. In view of the fact that theoretically it may be expected that the isoelectric point of lecithin and that of cephalin should occur at different magnitudes of pH, and in view of the fact that the material in the hands of Feinschmidt was undoubtedly a mixture of phosphatides, it is important to re-determine the isoelectric points of phosphatides on pure materials. Of considerable interest from both practical and theoretical viewpoint is the observation of Feinschmidt that lecithin may combine with protein and that the pH value of the isoelectric points of the resulting complex differs from that of either of the original materials. This conclusion also needs to be substantiated by more careful experimentation.

IV. Biological Aspect of the Phosphatides.

The interest of the biologist in phosphatides lies not in the theories of its structure, but in the part the substances play in the phenomena of life. The investigator even at the end of the eighteenth and of the early nineteenth centuries was attracted not only by the purely chemical side of the work, but also by its possible bearing on many fundamental problems of life. As times and knowledge progressed, the phosphatides were called upon to unravel the mystery of diverse biological phenomena, so that today there has accumulated a volume of literature on the subject too large for one man to digest even were he endowed with extraordinary abilities. The present writer has watched the biological work on phosphatides from a distance and hence could see only the currents of thought contained in it. Such as they seem to him they will be pointed out here. A few of them will be analyzed from the viewpoint of chemical theory, while some will be simply referred to.

The earlier worker on phosphatides was interested in them principally because they seemed to him specific of the brain and nerve tissue. Through the chemical analysis of the brain and of other organs, in respect to their phosphatides, he hoped to penetrate into

the causes of mental activity and of the respective "quality" of the brain of persons endowed with different mental abilities. Thus the brains of the idiot and of the normal person were analyzed, also those of persons with various forms of insanity.

Writes Couerbe in 1834, "L'étude chimique de cette masse essentielle devra donc nécessairement fournir quelque chose de remarquable surtout si elle est faite sur divers cerveaux provenant d'individus aliénés, d'individus idiots, enfin d'individus à l'état normal." He then proceeds to report the following findings. The phosphorus content of normal individuals varies in the neighborhood of 2.5 per cent, that of idiots is about 1 to 1.5 per cent, and that of insane about 3 to 4.5 per cent. Hence he reaches the conclusion that the sensitiveness of the brain is intimately associated with its content in phosphatides.

Couerbe's trend of thought inspired many workers up to our own day. Waldemar Koch particularly has devoted much energy to the search for relationship between the composition of brain phosphatides and the physiological or pathological condition of that organ.

Work of this nature was justified, especially at the end of the eighteenth and the early nineteenth centuries, when modern theories of solutions, of diffusion and osmosis, of potential differences in solutions, etc., did not exist, and when elementary composition was the principal characteristic of a substance which the chemist could deal with intelligently. Of more modern nature is the work which attempts to find an answer to the question of the biological function of phosphatides on the basis of either their chemical structure or of their physico-chemical properties.

The details of the current of thought which connects chemical structure and biological activity are the following. If in a series of organs with distinct function, or in the same organs of animals of different species, the phosphatides possess a distinct chemical structure, then it may be assumed that the phosphatides play a part in bringing about the specificity of the organ or of the species. Such a part might have been claimed for phosphatides on the basis of the work of S. Fränkel and his co-workers who were led to believe that phosphatides differed from organ to organ and from species to species. However, the various substances described by workers in Fränkel's

laboratory did not stand the test of more careful analysis, and as was stated in preceding sections, modern work tends to the view that the number of phosphatides is limited to three: lecithin, cephalin and sphingomyelin. In regard to lecithin, there exists undisputed evidence that the one derived from the liver differs in the details of its structure from that of the brain and of the egg yolk. The extent of the variations in other organs is not yet known. Theoretically, cephalin and sphingomyelin also permit of variations, but as yet there is no evidence of the existence of such. Thus at present it would be premature to formulate general conclusions as to the biological rôle of phosphatides on the basis of their chemical variability.

On the other hand, there exists evidence that cells of different organs differ in the proportions of total phosphatides contained in them. Nerking called attention to this fact in 1908. In 1913 and 1914 A. Mayer and Schaeffer came to the conclusion that the content of phosphatides is a constant for every cell or tissue and apparently is independent of the physiological factors such as growth or state of nutrition. The latter condition affects the content of phosphatides exclusively in the liver and then in a slight degree only. This finding is very significant inasmuch as it may be used as a basis for the theory that biological specificity, individuality, or character, is brought about not by single substances but rather by definite and constant equilibria of substances. The mechanism by which such equilibria are maintained is as yet unknown.

The universal distribution of phosphatides, their constant concentration in the same cells under differing conditions, creates the belief that phosphatides play a very important rôle in the organization of the living cell, even if they are not responsible for cell specificity. The explicit function of phosphatides was therefore the subject of much speculation by both the older and modern writers. Physiologist, morphologist and physical chemist have theorized on the subject, each from his own angle of vision. The general physiologist associated lecithin, or more correctly mixed phosphatides, with the function of tissue oxidations. Thunberg and A. H. Warburg followed the rate of oxidation of lecithin in the presence of iron, and were inclined to associate cell oxidation with phosphatides. Other workers, as Kovliova, saw in phosphatides substances which acted catalytically on the

oxidative processes of the animal organism. In the same sense may be viewed the conclusions of Vernon that the action of animal oxidases is dependent on the presence of phosphatides. Suggestive as the work on the rôle of phosphatides on the oxidative processes may be, one must not overlook the fact that the conclusions reached through it are rather of a speculative nature and are not the only possible and binding experimental conclusions.

More modern, definite and exact is the work which deals with that biological significance of phosphatides based on their physico-chemical properties. It has already been stated that phosphatides are ampholites with an isoelectric point at a definite hydrogen ion concentration, also that they are comparatively only slightly soluble in water. When they are immersed in an aqueous solution of electrolytes, a heterogeneous system is established. In the two principal phases of this system salts and their ions are distributed in different concentrations. The mechanism which brings about this difference in concentration is perhaps not very simple. Loeb and Beutner who were the first to interest themselves in this aspect of the phosphatide problem explained the phenomenon on the basis of differences in the solubility of the ions in water and in phosphatides. In the light of the more recent work of Loeb, the phenomenon of "membrane equilibrium" discovered by Donnan may play a more important, if not exclusive part in bringing about a difference in the ionic concentration within and without the phosphatide phase. This difference of concentration in its turn results in a difference of electric potentials which follows Nernst's law. Thus Loeb and Beutner, and Loeb, have shown that the so-called bioelectric currents are brought about in part by the physico-chemical properties of the phosphatides. We say advisedly "in part" for the reason that from the most recent work of Loeb, it follows that proteins share an equal rôle in the mechanism of bioelectric currents.

In this connection must be mentioned a very ingenious suggestion concerning the rôle of phosphatides made by Mulon. According to this writer phosphatides are present in mitochondria. In these morphological units the function of phosphatides is to establish a certain equilibrium of electrolytes within and without them.

Before concluding the general physiology of phosphatides, it is well to refer to the work dealing with the synthesis and mode of catabolism of phosphatides within the organism. The advance in the knowledge of this branch of the subject has been made through the work on the developing organism. Strange as it may seem, the study of the changes in the arrangements of the various phosphorus compounds in the developing egg brought out not the mechanism of the synthesis of lecithin, but of its disintegration. From the fact that in course of development the content of phosphatides declines, whereas the proportion of inorganic phosphatides and of nucleins rises, it may be concluded that lecithin in part serves for storing the phosphoric acid to be released for other purposes. The chemical facts underlying this thought seem to be well established by a number of investigators: Kossell, Maxwell, Mesernitzy, Schasel, Carpeaux, Plimmer and Scott, Robert and Wasteneys. Naturally, the fact that the reserve lecithin is broken down in course of development does not argue against the possibility that out of the debris new lecithin is formed in the young cells of the embryo. The limitations of the present day methods make it impossible to discover such a process even though it exists. On the other hand real synthesis of lecithin, it is claimed, occurs even in the adult. The foundation for this belief is the following. It was demonstrated by Plimmer that intestinal mucosa contains an enzyme capable of hydrolyzing lecithin, and also that hens fed on food containing a minimal proportion of lecithin lay eggs normal in respect to their phosphatide content. The most reliable work in this connection was done by McCollum and his co-workers, and by Fingerling. Also Smith and Mair have contributed in the work pointing toward this theory. There is one possible objection to the conclusion of McCollum and other workers which they have failed to take into account. The phosphatides which they regard as newly formed may in reality only be transported from other organs. This objection could be met if it were shown that hens could lay normal eggs indefinitely if maintained on food containing phosphoric acid only in form of its inorganic salt. A more definite proof for the assumption of phosphatide synthesis in the adult organism was furnished by MacLean and Williams (1909), who observed that the character of the fatty acids of the lecithin of eggs varies with the change of diet.

Passing from general to special physiology, one finds two chapters in which extensive reference was made to phosphatides. One is on fat metabolism and the other on blood coagulation.

In connection with the problem of fatty acid catabolism, a theory has been advanced which holds that fatty acids in the organism prior to their combustion enter into a complex molecule which by their entrance constitutes lecithin. This theory postulates that for some reasons chemical or physical, fatty acids are more easily oxidized by the organism when they are combined with glycerophosphoric acid than when they are in a free state. Loew theorized on this subject in 1899. Later, McCollum and his co-workers, then MacLean and Williams, and still later Bloor, furnished experimental evidence supporting the theory. In harmony also with this theory is the finding of Levene and Ingvaldsen and of Levene and Simms, that liver lecithin contains fatty acids of a higher degree of unsaturation than that of other organs. It is too early to predict the fate of this theory. However, its analogy with the theory of the oxidation of carbohydrates in the living organism is striking. Here also it is claimed that prior to its oxidation the sugar molecule enters into union with phosphoric acid, and that thus united it is more susceptible to the action of glycolytic enzymes. Both theories offer an attractive field for further work.

In all theories on blood coagulation since the days of Alexander Schmidt, an important place was attributed to substances which are now designated as "lipoids." In recent years an attempt was made to single out that one of the various "lipoids" which participates in the process of blood coagulation. This task was undertaken by Howell in 1912. He arrived at the conclusion that only cephalin possessed thromboplastic properties. Later in 1917, McLean, a student of Howell, connected the activity of cephalin with the state of unsaturation of one of the fatty acids present in its molecule. In the publication of 1912, Howell considers the possibility of the active principle (thromboplastic substances) being an impurity adhering to the phosphatide, and rules this assumption out on the basis of his experience. The finding of Howell is very important. To the knowledge of the writer it is the first instance where a definite physiological function was correlated with an individual phosphatide. The

very important observation of Delezenne and Fourneau of a similar nature will be referred to later.

To the writer it seems that further work is required before the actual part of cephalin in the process of blood coagulation will be made clear. According to Howell and other workers, egg lecithin has no thromboplastic action. In the light of our present knowledge "lecithin" as generally used is a mixture of undecomposed lecithin and cephalin. "Cephalin," on the other hand, contains all that is contained in "lecithin" and in addition decomposition products of lecithin and of cephalin, together with other impurities. Such was the material prepared by Howell. The purified material used by McLean and furnished by Levene and West was free from lecithin and its fragments, and was composed of cephalin, its decomposition products and perhaps some other impurities. The fact is significant that partially reduced cephalin which contained fewer impurities than the original material was inactive even though it contained part of its unsaturated radicles unaltered.

Thus the questions that arise are: Is lecithin in its thromboplastic action antagonistic to cephalin? Is a degree of decomposition required to render cephalin active, and if so, what degree? Finally, is thromboplastic activity due to something other than cephalin but adhering to it? These problems will be solved only after the methods of preparation of pure cephalin have been perfected.

Many other chapters of physiology deal with the phosphatides. As already mentioned, the references are too numerous to be discussed analytically and critically in a short review. On the other hand, they interest principally the specialist and not the one seeking general information. The reviewer begs the indulgence of the authors whose work he has failed to discuss.

Before concluding the review he wishes, however, to make a few remarks in connection with the rôle of lipoids in pathology and in clinical medicine. Great importance has been attributed to the distribution of phosphatides in the various organs and particularly in the blood in certain pathological conditions. The value of such work depends entirely on the degree of exactness of the analytical methods employed for this purpose. Bloor and Greenwald have contributed a great deal to place the problem on a more modern basis.

To those who are interested in work on immunity, or in work on the physiological action of phosphatides as chemical individuals, the reviewer desires once more to point out the importance of the observations of Delezenne and Fourneau on hemolysis, and those of MacLean on vitamines. Delezenne and Fourneau have demonstrated, as Manwaring suggested before them, that in hemolysis a cleavage product of lecithin is the active agent. Hence, when the activity of a phosphatide is tested, one must distinguish between the pure intact phosphatide and a mixture containing besides the phosphatide, also the products of its partial disintegration.

The work of MacLean has demonstrated the extreme caution which one has to exercise in regard to conclusions concerning the physiological activity of such substances as phosphatides. It had been claimed that lecithin could function as a vitamine. MacLean has proven that it functioned in this manner only when impure, and that it lost its vitamine activity when purified. It is a very difficult and laborious process to purify phosphatides, but for pharmacological and immunity work only pure substances should be employed.

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AROMATIC ARSENIC COMPOUNDS.

IX. Diazo-Amino Compounds of Arsanilic Acid and Its Derivatives.

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In a former communication[1] there was outlined a plan for the synthesis of aromatic arsenic compounds for chemotherapeutic study in which, among other series of substances which have since been published, were mentioned the two groups of diazo-amino and azo derivatives obtained from arsanilic acid. These substances were the first which we prepared for these investigations, and although interesting results were obtained with individual compounds in the treatment of experimental trypanosomiasis, we were soon led to discontinue the study of these types because of disadvantages which seemed inherent in them, such as the general instability of the diazo-amino compounds and certain harmful toxic effects induced by both groups. We believe, however, that a presentation of our chemical studies with these compounds should not be devoid of interest.

In their determination of the constitution of arsanilic acid Ehrlich and Bertheim[2] found that the amino group could be readily diazotized and that the resulting diazo compound coupled without difficulty, yielding azo dyes. We have found that this reaction could be extended to the preparation of well-defined diazo-amino compounds containing the arsonic acid residue. We have prepared several groups of such substances, using as couplers dialkylamines, simple arylamines, aminobenzoic acids, arylglycines, and amino-phenoxy-acetic acids.

The coupling of the secondary aliphatic amines and the simpler primary aromatic amines with diazotized arsanilic acid offered as a

[1] This Journal, 41, 1581 (1919).

[2] Ehrlich and Bertheim, Ber., 40, 3292 (1907).

rule but little difficulty the procedure being that adopted in the past for analogous substances. The reaction mixture was kept neutral or acid only with a weak acid such as acetic acid. The isolation of the individual compounds was accomplished either as the salt or as the acid, depending upon manipulative convenience. In certain cases it was found preferable, however, to diazotize the amino compound first and then to add arsanilic acid as the coupler. In the case of the aromatic amino acids, although coupling usually proceeded smoothly, it required considerable experimentation to determine in what form and under what conditions the product could best be isolated, since there were available the free acid, the monosodium salt, and the disodium salt from which to choose. The simpler aminobenzoic acids yielded diazo compounds under carefully controlled conditions by using the amino acid itself as coupler and isolating the products as the monosodium salts. In the first experiments in which *p*-amino-phenoxyacetic acid and its homologs were employed, however, it developed that the reaction either did not proceed entirely in the desired sense, or that the diazo-amino compounds possessed properties which rendered difficult their isolation from the reaction mixture. In such cases it was found that the amino acid esters coupled smoothly, giving the diazo-amino esters in excellent yield. Owing to the stability of the diazo-amino group on the alkaline side it then proved relatively simple to saponify the esters to the diazo-amino acids themselves. This method was consequently employed as a more certain means of obtaining these acids in satisfactory form and yield from the more complex amino-aryl acids, although in some instances the direct method might have been employed. This device also proved necessary in the preparation of the diazo-amino compound from *p*-aminophenol. Here *p*-aminophenyl benzoate was diazotized and coupled with arsanilic acid, yielding the diazo-amino benzoate, which was subsequently saponified.

The diazo-amino compounds derived from the secondary aliphatic amines and from most of the aromatic amino compounds formed both crystalline acids and sodium salts, the former possessing definite decomposition points and being as a rule difficultly soluble in the usual solvents, while the salts formed more or less yellow solutions in water. The acids were unstable in acid solution, readily dissociating

into the component amino and diazo compounds and evolving nitrogen on warming. In the case of several of the diazo-amino compounds derived from the arylglycines and the methyl derivatives of *p*-aminophenoxyacetic acid, it was found impossible to isolate the free acids owing to their slight tendency to crystallize and to the ease with which they dissociated in acid solution. On the other hand, the esters of these substances, with few exceptions, proved to be more stable and easy to isolate. Practically all of the diazo-amino sodium salts were readily soluble in water, the solutions giving immediate precipitates with the heavy metal ions.

On the whole the work with this class of substances proved difficult, since in each case the conditions best suited for the preparation and isolation of the particular compound had to be carefully studied. It was also found essential to work as rapidly as possible throughout, and at low temperatures in order to avoid the decomposition which otherwise readily occurred.

Owing to the mass of detail the work is presented practically in abstract form, in order not to use undue space.

EXPERIMENTAL.

The values given for water of crystallization were obtained with substances which had been dried to constant weight in the air. Water was determined as a rule by drying *in vacuo* at room temperature over sulfuric acid, since higher temperatures caused decomposition.

In order to avoid repetition, it will be understood that the diazotization of arsanilic acid was carried out as follows. Arsanilic acid was dissolved in 2 molecular equivalents of *N* hydrochloric acid, chilled to about 5° and diazotized with 1 equivalent of 5 *N* sodium nitrite solution. The reaction proceeded smoothly and completely and toward the end there was a pronounced color change from orange to pale yellow. The solution was then neutralized before coupling according to the method given under each compound to be described. The expression A→B indicates that the diazotized arsanilic acid solution was added to the coupler; B→A, that the coupler was added to the diazotized arsanilic acid.

In cases in which no acid group was present in addition to the arsonic acid residue, acetic acid was in all cases sufficient to liberate

the diazo-amino arsonic acid from solutions of the sodium salt. It was also found advantageous to work at rather high dilutions, except in the case of the aliphatic amines. When an additional carboxy group was present it was found necessary to add to the chilled solutions of the salt sufficient hydrochloric acid to render the reaction just acid to congo red.

(A) DIAZO-AMINO COMPOUNDS OF ARSANILIC ACID AND ALIPHATIC AMINES.

Diazobenzene - (4-arsonic acid) - dimethylamine.—B→neutralized A, coupling being complete in about 15 minutes. After adding acetic acid the product was recrystallized from alcohol, forming orange spears which intumesced at 182°. It is very difficultly soluble in cold alcohol, more readily in methyl alcohol, and decomposes in boiling water with nitrogen evolution.

Analyses. Calc. for $C_8H_{12}O_3N_3As$: N, 15.38; As, 27.44. Found: N, 15.52; As, 27.13.

On treating an alcoholic suspension with 1 mol. of 25% sodium hydroxide solution, heating, and carefully adding water until clear, the sodium salt crystallized on cooling, separating from 85% alcohol as glistening, salmon-colored platelets which dissolve easily in water.

Analyses. Calc. for $C_8H_{11}O_3N_3AsNa.3.5H_2O$: H_2O, 17.60. Found: 17.89.

Calc. for $C_8H_{11}O_3N_3AsNa$: N, 14.24. Found: 14.42.

Diazobenzene - (4-arsonic acid) - diethylamine.—The crude acid was reprecipitated from dil. sodium carbonate, dissolved in warm 50% alcohol, and cautiously diluted with an equal volume of water, separating as delicate, flat, cream-colored needles, which, when anhydrous, gradually soften and darken above 120°, decompose at about 195–200°, and dissolve readily in methyl alcohol and acetic acid.

Analyses. Calc. for $C_{10}H_{16}O_3N_3As.H_2O$: H_2O, 5.65. Found: 6.51.

Calc. for $C_{10}H_{16}O_3N_3As$: N, 13.96; As, 24.88. Found: N, 13.59; As, 25.12.

A neutral aqueous solution of the **sodium salt** was prepared, evaporated to dryness *in vacuo*, and the residue dissolved in a little hot 85% alcohol, the salt slowly crystallizing in the cold as rosets of somewhat deliquescent cream-colored needles.

Analyses. Calc. for $C_{10}H_{15}O_3N_3AsNa$: N, 13.00; As, 23.21. Found: N, 13.02; As, 23.82.

Diazobenzene - (4-arsonic acid) - piperidine.—The free acid, obtained from its salt, formed pale drab spherules and rosets of narrow, microscopic platelets which melt and effervesce at 162–3°, and are appreciably soluble in methyl and ethyl alcohol.

Analyses. Calc. for $C_{11}H_{16}O_3N_3As$: N, 13.42; As, 23.93. Found: N, 13.53; As, 23.69.

Separation of the **sodium salt** from the initial reaction mixture was facilitated by the addition of sodium acetate. It forms drab-colored, minute platelets from 85% alcohol.

Analyses. Calc. for $C_{11}H_{15}O_3N_3AsNa.3.5H_2O$: H_2O, 15.83. Found: 15.35.

Calc. for $C_{11}H_{15}O_3N_3AsNa$: N, 12.53. Found: 12.57.

Bis-diazobenzene - (4-arsonic acid) - pentamethylene-tetramine.—8.7 g. of arsanilic acid were diazotized with 40 cc. of *N* sodium nitrite solution, diluted with an equal volume of saturated sodium acetate solution, and then treated with 3 g. of hexamethylene-tetramine.[3] Coupling occurred rapidly with cleavage of formaldehyde, and 8 g. of the **sodium salt** quickly crystallized. Rapid recrystallization from water yielded lustrous, colorless platelets soluble in about 20 parts of water at ordinary temperatures.

Analyses. Calc. for $C_{17}H_{20}O_6N_8As_2Na_2.6H_2O$: H_2O, 14.68. Found: 14.31.

Calc. for $C_{17}H_{20}O_6N_8As_2Na_2$: N, 17.83. Found: 17.65.

On slowly treating a very dilute solution in warm water with acetic acid the **free acid** separates as narrow, minute, colorless, microscopic platelets which are practically insoluble in neutral solvents. It puffs up at 210–12°, and dissolves very slowly in boiling water with nitrogen evolution decomposition occurring rapidly on addition of a drop of acid, when the odor of formaldehyde becomes quite noticeable.

Analyses. Calc. for $C_{17}H_{22}O_6N_8As_2$: N, 19.18; As, 25.66. Found: N, 19.15; As, 25.40.

(B) DIAZO-AMINO COMPOUNDS OF ARSANILIC ACID AND THE SIMPLER AROMATIC AMINES.

Diazobenzene - (4-arsonic acid) - aniline.—Alcoholic B→neutralized A. The yellow sodium salt gradually crystallized on adding sodium acetate, separating from 85% alcohol as minute, orange platelets and flat needles which dissolve readily in water.

Analyses. Calc. for $C_{12}H_{11}O_3N_3AsNa.4H_2O$: H_2O, 17.35. Found: 17.02.

Calc. for $C_{12}H_{11}O_3N_3AsNa$: N, 12.24. Found: 12.21.

The **free acid** forms bundles of minute, yellow, microscopic needles which melt and then decompose at 112–13°. It is difficultly soluble in cold methyl and ethyl alcohols but quite readily on boiling, and decomposes in boiling water.

Analyses. Calc. for $C_{12}H_{12}O_3N_3As$: N, 13.08; As, 23.33. Found: N, 12.77; As 23.55.

Diazobenzene - (4-arsonic acid) - methylaniline.—Alcoholic B→A + sodium acetate yielded a brown, gummy precipitate which crystallized on rubbing. On dissolving in very dilute aqueous ammonia, adding an equal volume of alcohol, cooling, and acidifying with acetic acid, the pure acid separated as orange-brown aggregates of minute radiating needles, effervescing at 160–2° with preliminary

[3] Cf. Duden and Scharf, *Ann.*, **288**, 218 (1895).

reddening. It dissolves in dil. acid with an orange-red color appearing a faint purple in thin layers, apparently due to partial dye formation, although the solution still evolves nitrogen on heating.

Analyses. Calc. for $C_{13}H_{14}O_3N_3As$: N, 12.54. Found: 12.58.

When the diazo solution was neutralized with sodium hydroxide and then coupled with methylaniline, a deep brown-orange solution resulted from which the **sodium salt** separated on adding sodium acetate, as a thick paste of lustrous, orange platelets which were recrystallized from water.

Analyses. Calc. for $C_{12}H_{13}O_3N_3AsNa.5.5H_2O$: H_2O, 21.70. Found: 21.53.

Calc. for $C_{13}H_{13}O_3N_3AsNa$: N, 11.76; As, 21.00. Found: N, 11.83; As, 21.30.

Diazobenzene - (4-arsonic acid) - *p* - toluidine.—The sparingly soluble **sodium salt** separated in almost quantitative yield, forming yellow, glistening leaflets from water containing a few drops of dil. sodium carbonate.

Analyses. Calc. for $C_{13}H_{13}O_3N_3AsNa.6H_2O$: H_2O, 23.23. Found: 22.79.

Calc. for $C_{13}H_{13}O_3N_3AsNa$: N, 11.76; As, 20.99. Found: N, 11.78; As, 20.79.

The **free acid** is precipitated from a 50% alcoholic solution of the salt as thin, pale yellow, microscopic needles which intumesce at 130–2° and dissolve in hot alcohol.

Analysis. Calc. for $C_{13}H_{14}O_3N_3As$: N, 12.53. Found: 12.63.

Diazobenzene - (4-arsonic acid) - 4′ - chloro-aniline.—B + dil. hydrochloric acid → A + sodium acetate. The acid immediately precipitated in quantitative yield and gradually crystallized. Dissolved in much 50% alcohol with the aid of sodium hydroxide and re-acidified it forms an emulsion which rapidly changes on rubbing to hexagonal platelets which intumesce at 177° with preliminary darkening and are practically insoluble in neutral solvents.

Analysis. Calc. for $C_{12}H_{11}O_3N_3ClAs$: N, 11.82. Found: 12.08.

Dissolved in 1 mol. of dil. sodium hydroxide solution it yielded the **sodium salt** on addition of alcohol, forming almost colorless platelets from 50% alcohol.

Analyses. Calc. for $C_{12}H_{10}O_3N_3ClAsNa.2H_2O$: H_2O, 8.71. Found: 8.37.

Calc. for $C_{12}H_{10}O_3N_3ClAsNa$: N, 11.12; As, 19.86. Found: N, 11.20; As, 20.01.

Diazobenzene - (4-arsonic acid) - o - anisidine.—Although Jacobson and Hönigsberger[4] obtained a dye in using *o*-anisidine as a coupler, the method used in the above cases yielded the diazo-amino derivative. A certain amount of dye formation was, however, evident from the deep color. The sodium salt was salted out with sodium acetate and washed with alcohol. It separates from 85% alcohol as aggregates of brown, rectangular platelets dissolving readily in water.

Analyses. Calc. for $C_{13}H_{13}O_4N_3AsNa.5H_2O$: H_2O, 19.44. Found: 20.02.

Calc. for $C_{13}H_{13}O_4N_3AsNa$: N, 11.26. Found: 11.19.

The free acid was precipitated from a chilled solution of the salt in 50% alcohol as sheaves of flat, microscopic needles which, when anhydrous, sinter, then darken, and finally intumesce at 95–9°.

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[4] Jacobson and Hönigsberger, *Ber.*, 36, 4096 (1903).

Analyses. Calc. for $C_{13}H_{14}O_4N_3As.2.5H_2O$: H_2O, 11.37. Found: 10.58.

Calc. for $C_{13}H_{14}O_4N_3As$: N, 11.97. Found: 12.10.

Diazobenzene - (4-arsonic acid) - *p*-anisidine.—The **sodium salt** separates from 50% **alcohol** as rosets of pale brown leaflets.

Analyses. Calc. for $C_{13}H_{13}O_4N_3AsNa.5H_2O$: H_2O, 19.44. Found: 19.55.

Calc. for $C_{13}H_{13}O_4N_3AsNa$: N, 11.26; As, 20.08. Found: N, 11.39; As, 20.01.

The free acid was precipitated from a 33% alcoholic solution of the salt as pale brown aggregates of microscopic leaflets which sinter and begin to decompose at about 110° and intumesce slowly at 116–9° when anhydrous, and are quite soluble in methyl alcohol.

Analyses. Calc. for $C_{13}H_{14}O_4N_3As.0.5H_2O$: H_2O, 2.50. Found: 2.36.

Calc. for $C_{13}H_{14}O_4N_3As$: N, 11.97. Found: 11.88.

Diazobenzene - (4-arsonic acid) - 4′ - amino-acetanilide.—The **sodium salt** separated from 50% alcohol as plumes of tan, microscopic leaflets.

Analyses. Calc. for $C_{14}H_{14}O_4N_4AsNa.5H_2O$: H_2O, 18.36. Found: 17.38.

Calc. for $C_{14}H_{14}O_4N_4AsNa$: N, 14.00; As, 18.74; Found: N, 13.88; As, 18.49.

The free acid liberated from the salt in 50% alcohol forms long, drab, lustrous hexagonal, microscopic platelets, which decompose at 165–70° when anhydrous.

Analyses. Calc. for $C_{14}H_{15}O_4N_4As.H_2O$: H_2O, 4.54. Found: 4.80.

Calc. for $C_{14}H_{15}O_4N_4As$: N, 14.81. Found: 14.79.

Diazobenzene - (4-arsonic acid) - 4′ - aminophenyl benzoate.—6.4 g. of well powdered *p*-aminophenyl benzoate[5] suspended in 60 cc. of *N* hydrochloric acid were slowly diazotized at 10° with 2.2 g. of sodium nitrite. After 15 minutes additional turbining the solution was filtered from a small amount of undissolved material and then treated, with stirring, with 6.6 g. of arsanilic acid in 30 cc. of *N* sodium hydroxide solution. The light yellow, pasty diazo-amino compound solidified completely on standing overnight in the ice-box. The yield was 12 g. A portion, dissolved in a large volume of warm methyl alcohol and cautiously treated with water until crystallization began on rubbing, formed thin, yellow, microscopic leaflets which decompose at 155–8° and are practically insoluble in neutral solvents except hot methyl and ethyl alcohols.

Analysis. Calc. for $C_{19}H_{16}O_5N_3As$: N, 9.52. Found: 9.65.

Diazobenzene - (4-arsonic acid) - 4′ - aminophenol.—The crude benzoate was dissolved in 4 equivalents of 2 *N* sodium hydroxide solution, allowed to stand for 15 minutes at room temperature, saturated with sodium acetate, and then chilled and neutralized with acetic acid. On rubbing the **sodium salt** slowly crystallized, separating from 85% alcohol as rosets of yellow microscopic needles. Under carefully controlled conditions acetic acid precipitated the microcrystalline acid which, however, was too unstable to isolate.

Analyses. Calc. for $C_{12}H_{11}O_4N_3AsNa.4H_2O$: H_2O, 16.69. Found: 16.38.

Calc. for $C_{12}H_{11}O_4N_3AsNa$: N, 11.69; As, 20.87. Found: N, 11.34; As, 20.76.

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[5] Cf. Wohl, *Ber.*, **36**, 4143 (1903).

Diazobenzene - (4-arsonic acid) - 4′ - amino-acetophenone.—A + 1 mol. alkali →diazotized B + sodium acetate. The resulting **sodium salt**, recrystallized from 85% alcohol, formed brownish-yellow rosets of minute, flat needles.

Analyses. Calc. for $C_{14}H_{13}O_4N_3AsNa.6H_2O$: H_2O, 21.91. Found: 21.51.

Calc. for $C_{14}H_{13}O_4N_3AsNa$: N, 10.91. Found: 10.94.

The **free acid** consists of sheaves of bright yellow, microscopic needles which intumesce to a reddish mass at 177–8° after preliminary darkening and sintering, and dissolve in boiling methyl alcohol.

Analyses. Calc. for $C_{14}H_{14}O_4N_3As$: N, 11.57; As, 20.63. Found: N, 11.40; As, 20.91.

(c) DIAZO-AMINO COMPOUNDS OF ARSANILIC ACID AND THE AMINOBENZOIC ACIDS.

Diazobenzene - (4-Arsonic Acid) - 2′ - Aminobenzoic Acid.—Only the following conditions were found to yield this substance and its salts in pure form. 8.7 g. of arsanilic acid diazotized in 80 cc. of *N* hydrochloric acid were treated with 80 cc. of saturated sodium acetate solution and then with 5.5 g. of anthranilic acid dissolved in 80 cc. of *N* sodium hydroxide solution. The **monosodium salt** soon separated in almost quantitative yield as a brownish-yellow powder. On dissolving in dil. sodium hydroxide solution, adding sodium acetate and then a slight excess of acetic acid, a gelatinous mass formed which rapidly changed to radiating masses of flat, yellow microscopic needles of the monosodium salt. The substance is difficultly soluble in cold water.

Analyses. Calc. for $C_{13}H_{11}O_5N_3AsNa.4.5H_2O$: H_2O, 17.31. Found: 16.74.

Calc. for $C_{13}H_{11}O_5N_3AsNa$: N, 10.85. Found: 11.06.

A concentrated solution prepared with an additional mol. of sodium hydroxide yielded with sodium acetate the yellow **disodium salt** which crystallized from 85% alcohol as long, flat, yellow needles, dissolving easily in water with a yellow color.

Analyses. Calc. for $C_{13}H_{10}O_5N_3AsNa_2.H_2O$: H_2O, 26.05. Found: 26.37.

Calc. for $C_{13}H_{10}O_5N_3AsNa_2$: N, 10.26; As, 18.32. Found: N, 10.14; As, 17.94.

With a large excess of acetic acid, it immediately gives the **free acid** as thin, yellow microscopic needles which intumesce at 160°, and dissolve in hot methyl or ethyl alcohol.

Analysis. Calc. for $C_{13}H_{12}O_5N_3AS$: N, 11.51. Found: 11.51.

Diazobenzene - (4-Arsonic Acid) - 3′ - Aminobenzoic Acid.—The same conditions were used, except that the addition of 12 cc. of 50% acetic acid was necessary to cause the precipitation of the **monosodium salt.** Recrystallized as in the preceding preparation it forms a cream-colored, microcrystalline, anhydrous salt.

Analysis. Calc. for $C_{13}H_{11}O_5N_2AsNa$: N, 10.85. Found: 10.71.

An aqueous suspension was dissolved with just sufficient sodium hydroxide

and treated with absolute alcohol to incipient turbidity. The resulting **disodium salt** separated from 70% alcohol as long, thin, pale yellow, microscopic needles.

Analyses. Calc. for $C_{13}H_{10}O_5N_2AsNa_2.6.5H_2O$: H_2O, 22.24. Found: 21.68.

Calc. for $C_{13}H_{10}O_5N_2AsNa_2$: N, 10.26. Found: 10.32.

A well chilled solution in 50 parts of 50% alcohol was made just acid to congo red, the **arsonic acid** then separating as buff-colored microcrystalline rosets which intumesce at 141° and dissolve in hot methyl and ethyl alcohols.

Analyses. Calc. for $C_{13}H_{12}O_6N_2As$: N, 11.51; As, 20.52. Found: N, 11.29; As, 20.54.

Diazobenzene - (4-Arsonic Acid) - 4′ - Aminobenzoic Acid.—The special conditions necessary were treatment of the well chilled diazotate from 4.4 g. of arsanilic acid in 40 cc. of *N* hydrochloric acid with 60 cc. of alcohol and then rapidly with a solution of 2.8 g. of *p*-aminobenzoic acid in 10 cc. of 2 *N* sodium hydroxide solution. The deep brown solution deposited the yellow, **amorphous monosodium salt** almost at once and this changed to aggregates of microscopic needles under 50% alcohol. It was dissolved in 50% alcohol with a little sodium hydroxide and again acidified with acetic acid in slight excess, crystallizing in anhydrous form on rubbing. It is very sparingly soluble in cold water. The **free acid** formed flat, microscopic needles, but was not obtained pure.

Analyses. Calc. for $C_{13}H_{11}O_5N_3AsNa$: N, 10.85; As, 19.36. Found: N, 11.07; As, 19.23.

Diazobenzene - (4-arsonic acid)- 3′ - amino-6′-methoxybenzoic methyl ester.—B hydrochloride → A + sodium acetate. The resulting thick, gelatinous mass crystallized after adding one-half volume of alcohol and stirring and chilling. Dissolved in much cold alcohol and treated with an equal volume of water the ester acid crystallized as buff-colored aggregates of narrow platelets with notched ends, melting and evolving gas at 90–95° and dissolving readily in methyl or ethyl alcohol, especially on warming.

Analyses. Calc. for $C_{16}H_{18}O_6N_3As.3H_2O$: H_2O, 11.31. Found: 11.08.

Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 10.18.

Diazobenzene - (4-arsonic acid) - 3′ - amino-6′-methoxybenzoic acid.—The ester, warmed for one hour in 50% alcohol containing 3 mols. of sodium hydroxide, neutralizing with 1 mol. of acetic acid, and adding alcohol to incipient turbidity, gave the **disodium salt**, which separated from 70% alcohol as rosets of pale, brownish-yellow, hygroscopic needles.

Analysis. Calc. for $C_{14}H_{12}O_6N_3AsNa_2$: N, 9.57. Found: 9.67.

The **arsonic acid** was precipitated in 50% alcohol as rosets of yellow microscopic needles which decompose at 140° when anhydrous.

Analyses. Calc. for $C_{14}H_{14}O_6N_3As.1.5H_2O$: H_2O, 6.40. Found: 6.47.

Calc. for $C_{14}H_{14}O_6N_3As$: N, 10.63. Found: 10.64.

Diazobenzene - (4-arsonic acid) - 3′ - amino-anisic methyl ester.—Alcoholic B → A + sodium acetate. The ester acid, recrystallized from much 50% alcohol,

forms glistening yellow needles which decompose at about 150° with preliminary darkening, and dissolve readily in methyl alcohol.

Analyses. Calc. for $C_{16}H_{18}O_6N_3As.2.5H_2O$: H_2O, 9.91. Found: 9.20.

Calc. for $C_{16}H_{18}O_6N_3As$: N, 10.26. Found: 10.37.

Diazobenzene-(4-arsonic acid)-3′-amino-anisic acid.—I. Best prepared by saponification of the ester as described for the isomeric *o*-methoxy derivative. From 70% alcohol the **disodium salt** forms balls of fine yellow needles. II. The salt was also obtained directly, although in poorer yield, by the reaction of sodium 3-amino-anisate with the neutralized diazo solution and the addition of an equal volume of absolute alcohol.

Analyses. Calc. for $C_{14}H_{12}O_6N_3AsNa_2.10H_2O$: H_2O, 29.08. Found: I, 30.13; II, 30.36.

Calc. for $C_{14}H_{12}O_6N_3AsNa_2$: N, 9.57; As, 17.08. Found: N, I, 9.68; II, 9.69; As, 17.23, 16.92.

The **free acid** was precipitated from 50% alcohol as aggregates of minute, yellow, microscopic needles which intumesce at 150–5° with preliminary darkening when anhydrous and are difficultly soluble in hot methyl alcohol.

Analyses. Calc. for $C_{14}H_{14}O_6N_3As.1.5H_2O$: H_2O, 6.40. Found: 6.30.

Calc. for $C_{14}H_{14}O_6N_3As$: N, 10.63. Found: 10.63.

Diazobenzene-(4-arsonic acid)-6′-aminopiperonylic methyl ester.—B hydrochloride→A, followed by sodium acetate. Reprecipitated from very dilute aqueous ammonia the ester acid forms minute aggregates of short, flat, almost colorless needles which do not decompose below 280° and are practically insoluble in the neutral solvents.

Analysis. Calc. for $C_{15}H_{14}O_7N_3As$: N, 9.92. Found: 9.91.

On saponification, a sodium salt was easily obtained, which, however, gave poor analytical figures, as did also the acid obtained from it. An attempt to prepare the diazo-amino acid directly from 6-aminopiperonylic acid yielded a dye which was not investigated further.

Diazobenzene-(4-arsonic acid)-4′-aminocinnamic ethyl ester.—3.8 g. of amino ester were diazotized in 60 cc. of *N* hydrochloric acid with chilling and treated with 20 cc. of *N* sodium hydroxide solution. Without filtering from the yellow precipitate which formed, 4.4 g. of arsanilic acid in 20 cc. of *N* sodium hydroxide solution were added and finally 50 cc. of saturated sodium acetate solution. The initial amorphous precipitate changed to flat, yellow, microscopic needles of the **sodium salt**, which separates from 70% alcohol as bright yellow microscopic needles which are sparingly soluble in cold water.

Analyses. Calc. for $C_{17}H_{17}O_5N_3AsNa.6H_2O$: H_2O, 19.67. Found: 19.69.

Calc. for $C_{17}H_{17}O_5N_3AsNa$: N, 9.52. Found: 10.08.

The **arsonic acid** forms aggregates of fine, yellow, microscopic needles which decompose at 155–60°.

Analysis. Calc. for $C_{17}H_{18}O_5N_3As$: N, 10.02; As, 17.89. Found: N, 10.32; As, 17.88.

Diazobenzene - (4-arsonic acid) - 4′ - aminocinnamic acid.—The ester salt was saponified in 50% alcohol containing 2 mols. of sodium hydroxide, followed by 1 mol. of acetic acid, and then alcohol. Recrystallized from 70% alcohol the **disodium salt** formed yellow microscopic needles which dissolved readily in water.

Analyses. Calc. for $C_{15}H_{12}O_5N_3AsNa_2.6H_2O$: H_2O, 19.89. Found: 19.96.

Calc. for $C_{15}H_{12}O_5N_3AsNa_2$: N, 9.66; As, 17.23. Found: N, 9.43; As, 16.83.

The **acid** is precipitated from 50% alcohol as a gelatinous mass which changes to yellow, microscopic needles which decompose at 155–60° with preliminary darkening and sintering when anhydrous, and are appreciably soluble in boiling methyl alcohol.

Analyses. Calc. for $C_{15}H_{14}O_5N_3As.\ 0.5\ H_2O$: H_2O, 2.25. Found: 2.11.

Calc. for $C_{15}H_{14}O_5N_3As$: N, 10.74. Found: 10.83.

Diazobenzene - (4-arsonic acid) - 4′ - amino-phenylarsonic acid.—The diazo solution from 4.4 g. of arsanilic acid was treated with 4.4 g. of arsanilic acid in 40 cc. of *N* sodium hydroxide solution. The **monosodium salt** crystallized on rubbing. From 50% alcohol it forms thin, yellow, lustrous platelets which dissolve appreciably in water.

Analyses. Calc. for $C_{12}H_{12}O_6N_3As_2Na.2.5H_2O$: H_2O, 8.79. Found: 9.47.

Calc. for $C_{12}H_{12}O_6N_3As_2Na$: N, 8.99. Found: 9.01.

In 1 mol. of normal sodium hydroxide it yields the **disodium salt** on addition of sodium acetate, separating from 50% alcohol as woolly masses of delicate yellow needles which dissolve quite readily in water.

Analyses. Calc. for $C_{12}H_{11}O_6N_3As_2Na_2.7H_2O$: H_2O, 20.49. Found: 20.18.

Calc. for $C_{12}H_{11}O_6N_3As_2Na_2$: N, 8.59. Found: 8.61.

The **arsonic acid** precipitated from dilute aqueous solution, separates from 95% alcohol as pale yellow aggregates of microscopic needles which intumesce at 154° with preliminary darkening and sintering.

Analyses. Calc. for $C_{12}H_{13}O_6N_3As_2$: N, 9.44; As, 33.68. Found: N, 9.17; As, 33.84.

(D) DIAZO-AMINO COMPOUNDS OF ARSANILIC ACID WITH ARYL GLYCINES.

Diazobenzene - (4-arsonic acid) - phenylglycine.—Alcoholic B → neutralized A gave a thick slurry of the red-brown monosodium salt. The collected salt was dissolved in 10 parts of 50% alcohol with sodium hydroxide and the filtrate treated with a slight excess of acetic acid, slowly separating as flat, brown-yellow microscopic needles which are sparingly soluble in cold water. The free acid could not be obtained crystalline. The properties of the substance show that it is a true diazo-amino compound, but its color and that of its solutions indicate that it is contaminated with the corresponding azo dye, especially as Mai[6] obtained dyes on using phenylglycine as a coupler in mineral acid solution.

[6] Mai, *Ber.*, **35**, 580 (1902).

Analyses. Calc. for $C_{14}H_{13}O_5N_3AsNa.2H_2O$: H_2O, 8.24. Found: 7.21.

Calc. for $C_{14}H_{13}O_5N_3AsNa$: N, 10.47; As, 18.70. Found: N, 10.22; As, 18.90.

Diazobenzene - (4-arsonic acid) - *p* - tolylglycine.—B + 1 mol. alkali → A + sodium acetate, gave the **monosodium salt**, which was dissolved in dil. sodium hydroxide solution, treated with saturated sodium acetate solution, and then with an excess of acetic acid. The pure salt crystallized as radiating masses of tan, microscopic needles which are difficultly soluble in cold water.

Analyses. Calc. for $C_{15}H_{15}O_5N_3AsNa.1.5H_2O$: H_2O, 6.11. Found: 6.83.

Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.12; As, 18.06. Found: N, 10.07; As, 17.68.

When liberated in 50% alcohol the **acid** separates as radiating masses of flat, pale terra-cotta-colored microscopic needles which intumesce at 148–9° with preliminary softening and reddening, and are appreciably soluble in alcohol.

Analysis. Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69. Found: 10.77.

Diazobenzene - (4-arsonic acid) - benzylglycine.—B + mol. alkali → neutralized A followed by hydrochloric acid. The precipitate was dissolved in warm alcohol and water added to incipient turbidity, the arsonic acid separating as an almost colorless, glistening mass of branched and curved microscopic needles and prisms which intumesce at 155–60°. It is readily soluble in methyl alcohol.

Analyses. Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.68; As, 19.07. Found: N, 10.68; As, 19.05.

Diazobenzene - (4-arsonic acid) - 4′ - methoxy-phenylglycine ethyl ester.—Alcoholic B → neutralized A + equal volume of alcohol, followed by sodium acetate, gave the **sodium salt** which separates from 85% alcohol as yellow-brown leaflets and flat needles which are easily soluble in water. The acid could not be obtained crystalline.

Analyses. Calc. for $C_{17}H_{19}O_6N_3AsNa.4H_2O$: H_2O, 13.55. Found: 13.79.

Calc. for $C_7H_{19}O_6N_3AsNa$: N, 9.15. Found: 9.05.

Diazobenzene - (4-arsonic acid) - 4′ - methoxy-phenylglycine.—B + 1 mol. alkali → neutralized A, on chilling in a freezing mixture, treating with an excess of acetic acid, and rubbing, gave the **monosodium salt** as a highly colored powder. Redissolved in dil. sodium hydroxide solution and acidified with acetic acid, it separated as yellow, microscopic platelets which are sparingly soluble in cold water but dissolve on gentle warming, forming reddish solutions. The acid could not be made to crystallize. The salt was also obtained by saponification of the ethyl ester described above.

Analyses. Calc. for $C_{15}H_{15}O_6N_3AsNa.2H_2O$: H_2O, 7.71. Found: 6.98.

Calc. for $C_{15}H_{15}O_6N_3AsNa$: N, 9.74; As, 17.39. Found: N, 9.79; As, 17.16.

Diazobenzene - (4-arsonic acid) - 4′ - ethoxy-phenylglycine ethyl ester.—Obtained as in the preceding case, the **sodium salt** separates from 70% alcohol as lustrous, cream-colored leaflets.

Analyses. Calc. for $C_{18}H_{21}O_6N_3AsNa.2.5H_2O$: H_2O, 8.69. Found: 8.78.

Calc. for $C_{18}H_{21}O_6N_3AsNa$: N, 8.88. Found: 9.05.

Liberated from 25% alcoholic solution, the **acid** forms almost colorless microscopic rhombohedra.

Diazobenzene - (4-arsonic acid) - 4′ - ethoxy-phenylglycine.—On saponifying the ester in 2 mols. of *N* sodium hydroxide solution at room temperature, adding a little sodium acetate, and then an excess of acetic acid with chilling, the **monosodium salt** of the glycine arsonic acid crystallized as aggregates of thin, yellow microscopic needles which are sparingly soluble in water. The free acid could not be obtained crystalline.

Analyses. Calc. for $C_{16}H_{17}O_6N_3AsNa.2H_2O$: H_2O, 7.48. Found: 6.49.

Calc. for $C_{16}H_{17}O_6N_3AsNa$: N, 9.44; As, 16.85. Found: N, 9.54; As, 16.63.

This salt was also obtained directly from the diazo solution and *p*-ethoxy-phenylglycine, as in the case of the *p*-methoxy compound.

(E) DIAZO-AMINO COMPOUNDS OF THE AMINO-PHENOXYACETIC ACIDS

Diazobenzene - (4-arsonic acid) - 4′ - amino-phenoxyacetic ethyl ester.—B hydrochloride → neutralized A, half saturated with sodium acetate, gave the **sodium salt** which forms brownish aggregates of microscopic needles from 85% alcohol.

Analyses. Calc. for $C_{16}H_{17}O_6N_3AsNa.5H_2O$: H_2O, 16.83. Found: 17.04.

Calc. for $C_{16}H_{17}O_6N_3AsNa$: N, 9.44; As, 16.83. Found: N, 9.84; As, 16.99.

Liberated in 50% alcoholic solution, the **ester acid** forms salmon-colored, minute, glistening leaflets which melt and decompose at 132–3° with preliminary softening and dissolve readily in warm methyl and ethyl alcohols.

Analysis. Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 9.76.

Diazobenzene - (4-arsonic acid) - 4′ - amino-phenoxyacetic acid.—Although this substance was prepared directly from *p*-amino-phenoxyacetic acid[7] it was found preferable to saponify the above ester. The ester salt was warmed gently for one hour with 2 mols of *N* sodium hydroxide solution and an equal volume of alcohol, chilled, neutralized with acetic acid, and the crude **disodium salt** precipitated with alcohol. It forms brown needles from 85% alcohol which gradually darken and decompose on standing.

Analyses. Calc. for $C_{14}H_{12}O_6N_3AsNa_2.6.5H_2O$: H_2O, 21.05. Found: 21.35.

Calc. for $C_{14}H_{12}O_6N_3AsNa_2$: N, 9.56. As, 17.07. Found: N, 9.58; As, 17.06.

A well chilled solution in 100 parts of water was strongly acidified with acetic acid, causing the sudden incomplete separation of the free **arsonic acid** as aggregates of microscopic crystals. The cold mixture was just acidified to congo red and filtered cold, for if allowed to warm the acid rapidly dissociates and dissolves. It melts and intumesces at 132°.

Analysis. Calc. for $C_{14}H_{14}O_6N_3As$: N, 10.64. Found: 10.41.

Diazobenzene - (4-arsonic acid) - *p* - amino-phenoxyacetamide.—Aqueous *p*-amino-phenoxyacetamide[8] → neutralized A, half saturated with sodium acetate,

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[7] THIS JOURNAL, 39, 2196 (1917).

[8] *Ibid.*, 39, 2423 (1917).

gave the **sodium salt** in excellent yield. It separates from 50% alcohol as rosets and sheaves of delicate, drab-colored needles which dissolve fairly readily in water.

Analyses. Calc. for $C_{14}H_{14}O_5N_4AsNa.6H_2O$: H_2O, 20.62. Found: 19.88.

Calc. for $C_{14}H_{14}O_5N_4AsNa$: N, 13.46; As, 18.02. Found: N, 13.72; As, 17.56.

The **arsonic acid**, obtained from the salt in 50% alcohol, formed branched aggregates of cream-colored, microscopic needles and hairs which melt and effervesce at about 162° with preliminary browning.

Analysis. Calc. for $C_{14}H_{15}O_5N_4As$: N, 14.22. Found: 14.09.

Diazobenzene - (4-arsonic acid) - *p* - amino-phenoxyacet-methylamide.—The amorphous salt from *p*-amino-phenoxyacet-methylamide[9] soon crystallized, separating from water as pale yellow aggregates of hair-like needles.

Analyses. Calc. for $C_{15}H_{16}O_5N_4AsNa.4H_2O$: H_2O, 14.34. Found: 14.60.

Calc. for $C_{15}H_{16}O_5N_4AsNa$: N, 13.02; As, 17.43. Found: N, 12.94; As, 17.77.

The free **arsonic acid** from a dilute, 50% alcoholic solution of the salt, forms minute, flat, glistening cream-colored needles which melt with decomposition at 170° with preliminary darkening.

Analyses. Calc. for $C_{15}H_{17}O_5N_4As.H_2O$: H_2O, 4.23. Found: 3.66.

Calc. for $C_{15}H_{17}O_5N_4As$: N, 13.73. Found: 13.33.

Diazobenzene - (4-arsonic acid) - 4′ - methylamino-phenoxyacetic acid.—Coupled in neutral solution and then acidified strongly with acetic acid, *p-N*-methylamino-phenoxyacetic acid[10] gave the diazo-amino acid which on redissolving in dil. alkali and reprecipitating with acetic acid in excess, forms greenish-yellow aggregates of microscopic spindles which decompose at 155–60°.

Analyses. Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.26; As, 18.32. Found: N, 10.47; As, 18.50.

Diazobenzene - (4-arsonic acid)- 3′ - methyl-4′ -amino-phenoxyacetic methyl ester.—In neutral solution, in the presence of sodium acetate, the amino ester hydrochloride[11] gave the **sodium salt** as a thick mass of reddish-brown, microscopic needles. In 50% alcohol, acetic acid precipitates the **acid** as rosets of minute needles, melting and decomposing at 105–7°.

Analysis. Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 9.83.

Diazobenzene - (4-arsonic acid) - 3′ - methyl-4′-amino-phenoxyacetic acid.—The ester was saponified and the **disodium salt** isolated as in previous examples. Dissolved in a small volume of water and treated with alcohol it crystallized as light brown, glistening plates containing water of crystallization which was not determined. The free acid proved too unstable for isolation.

Analyses. Calc. for $C_{15}H_{14}O_6N_3AsNa_2$: N, 9.27; As, 16.55. Found: N, 9.04; As, 16.32.

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[9] *Loc. Cit.*, p. 2424.

[10] *Ibid.*, 39, 2197 (1917).

[11] *Loc. cit.*, p. 2201.

Diazobenzene - (4-arsonic acid)- 2′ - methyl-4′-amino-phenoxyacetic methyl ester.—The sodium salt, obtained from the amino ester hydrochloride,[12] gave with acetic acid in 50% alcohol the **acid** as aggregates of round, purple-brown, microscopic leaflets which decompose at 143–4° and dissolve in hot methyl and ethyl alcohols.

Analysis. Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 9.81.

Diazobenzene-(4-arsonic acid) - 2′ - methyl-4′-amino-phenoxyacetic acid.—The disodium salt obtained from the ester crystallized from dil. alcohol as easily soluble, long, flat, pinkish-yellow, microscopic needles which gradually decomposed on exposure. The acid proved to be too unstable for isolation.

Analyses. Calc. for $C_{15}H_{14}O_6N_3AsNa_2.4.5H_2O$: H_2O, 15.16. Found: 15.42.
Calc. for $C_{15}H_{14}O_6N_3AsNa_2$: N, 9.27; As, 16.55. Found: N, 9.50; As, 16.81.

Diazobenzene - (4-arsonic acid) - 2′, 5′ - dimethyl-4′-amino-phenoxyacetic methyl ester.—Amino ester hydrochloride[13] in 50% alcohol → A, followed by sodium acetate. Addition of an equal volume of alcohol to the thick amorphous slurry gave the micro-crystalline **ester acid** which was dissolved in warm 50% alcohol with ammonia, diluted with several volumes of alcohol, chilled, and acidified with acetic acid, crystallizing as gray-yellow aggregates of short, flat, pointed microscopic needles. It decomposes at about 120° and is quite soluble in hot methyl alcohol.

Analyses. Calc. for $C_{17}H_{20}O_6N_3As.1.5\ H_2O$: H_2O, 5.82. Found: 5.89.
Calc. for $C_{17}H_{20}O_6N_3As$: N, 9.61; As, 17.15. Found: N, 9.69; As, 17.35.

On saponifying the ester what was presumably the **disodium salt** of the acid was obtained as pale yellow needles but all attempts to obtain an analytically pure sample of the salt as well as of the free acid proved futile.

Diazobenzene - (4-arsonic acid) - 2′ - methyl - 5′ - *iso*propyl - 4′ -amino-phenoxyacetic methyl ester.—Amino ester hydrochloride[14] A → + sodium acetate, followed by alcohol gave the ester acid, which, reprecipitated from ammoniacal 50% alcoholic solution, formed microscopic needles which decompose at 145° and are appreciably soluble in hot alcohol.

Analysis. Calc. for $C_{19}H_{24}O_6N_3As$: N, 9.03. Found: 9.16.

Diazobenzene - (4-arsonic acid) - 2′ - methyl - 5′ - *iso*propyl - 4′ -amino-phenoxyacetic acid.—The ester was saponified as usual, yielding the **disodium salt.** Dissolved in a small volume of water and treated with alcohol it formed minute, flat, pale brown needles.

Analyses. Calc. for $C_{18}H_{20}O_6N_3AsNa_2.9.5H_2O$: H_2O, 25.68. Found: 25.39.
Calc. for $C_{18}H_{20}O_6N_3AsNa_2$: N, 8.49; As, 15.14. Found: N, 8.24; As, 15.05.
All attempts to obtain the free acid failed.

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[12] *Ibid.*, p. 2199.
[13] *Ibid.*, p. 2205.
[14] *Ibid.*, p. 2206.

Diazobenzene - (4-arsonic acid) - 3′ - methyl-6′-*iso*propyl-4′-amino-phenoxyacetic methyl ester.—Prepared from the ester hydrochloride[15] as in the case of the 2, 5-dimethyl analog. Recrystallized from 85% alcohol the acid formed rosets of yellow microscopic leaflets. When rapidly heated it intumesces to an orange mass at about 130°, but if slowly heated it turns orange above 120°, then gradually darkens, but does not melt below 275°.

Analyses. Calc. for $C_{19}H_{24}O_6N_3As.1.5H_2O$: H_2O, 5.49. Found: 4.56.

Calc. for $C_{19}H_{24}O_6N_3As$: N, 9.03. Found: 8.92.

Diazobenzene - (4-arsonic acid) - 3′ - methyl-6′-*iso*propyl-4′-amino-phenoxyacetic acid.—The crude **disodium salt** from the ester was reprecipitated from a little water by alcohol, separating as lustrous yellow microscopic leaflets.

Analyses. Calc. for $C_{18}H_{20}O_6N_3AsNa_2.4H_2O$: H_2O, 12.69. Found: 12.26.

Calc. for $C_{18}H_{20}O_6N_3AsNa_2$: N, 8.49; As, 15.14. Found: N, 8.36; As, 14.67.

The acid could not be isolated.

Diazobenzene-(4-arsonic acid) - 2′ - bromo-4′-amino-phenoxyacetic methyl ester.—The ester acid from 2-bromo-4-amino-phenoxyacetic methyl ester hydrochloride[16] was reprecipitated from very dilute aqueous ammonia, forming a jelly which changed to long, curved, cream-colored, microscopic needles which decompose at 154–5°.

Analysis. Calc. for $C_{15}H_{15}O_6N_3BrAs$: N, 8.61. Found: 8.71.

Diazobenzene - (4-arsonic acid) - 2′ - bromo-4′-amino-phenoxyacetic acid.—The **disodium salt** obtained from the ester was recrystallized as usual, forming flat, lustrous, pale brownish-yellow needles.

Analyses. Calc. for $C_{14}H_{11}O_6N_3BrAsNa_2.4H_2O$: H_2O, 12.20. Found: 12.44.

Calc. for $C_{14}H_{11}O_6N_3BrAsNa_2$: N, 8.11; As, 14.47. Found: N, 8.26; As, 14.71.

On acidifying an aqueous solution of the salt to congo red, a thick jelly formed, which, on addition of alcohol changed to delicate, pale yellow, microscopic needles which decompose at about 120° when anhydrous.

Analyses. Calc. for $C_{14}H_{13}O_6N_3BrAs.3H_2O$: H_2O, 10.22. Found: 9.41.

Calc. for $C_{14}H_{13}O_6N_3BrAs$: N, 8.86. Found: 9.03.

Diazobenzene - (4-arsonic acid) - 2 - methyl-4-amino-6-bromo-phenoxyacetic methyl ester.—Amino ester hydrochloride[17] in 50% alcohol neutralized→ A + sodium acetate. The yellow diazo-amino sodium salt, in ammoniacal dil. alcoholic solution, was acidified with acetic acid, yielding the free **ester arsonic acid** as bundles of delicate, pinkish, microscopic needles which intumesce at 188°.

Analyses. Calc. for $C_{16}H_{17}O_6N_3BrAs$: N, 8.37; As, 14.92. Found: N, 8.36; As, 15.15.

Diazobenzene - (4-arsonic acid) - 2 - methyl-4-amino-6-bromo-phenoxyacetic

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[15] *Loc. cit.*, p. 2207.

[16] *Ibid.*, p. 2209.

[17] *Ibid.*, 39, 2211 (1917).

acid.—The **disodium salt** was obtained as an indefinitely crystalline product and was dried *in vacuo*. It dissolves easily in water with a deep orange color.

Analyses. Calc. for $C_{15}H_{13}O_6N_3BrAsNa_2$: N, 7.90; As, 14.08. Found: N, 7.75; As, 13.42.

In dilute 50% alcoholic solution the salt yielded the **free acid** as grayish, microcrystalline aggregates which decompose at about 155°, with preliminary darkening and softening.

Diazobenzene-(4-arsonic acid) -4′ - amino-6′-aceto-phenoxyacetic methyl ester.—The methyl ester hydrochloride[18] ⟶ A + 1 mol. sodium hydroxide, followed by sodium acetate. The free acid, obtained from a dilute 50% alcoholic solution of the salt, was dissolved in 50% alcohol with a little aqueous ammonia and precipitated with a large excess of acetic acid, separating as rosets and sheaves of microscopic, often hair-like needles. It effervesces and reddens at about 155°, with preliminary darkening.

Analysis. Calc. for $C_{17}H_{18}N_7O_3As$: N, 9.31. Found: 9.64.

Diazobenzene- (4-arsonic acid) - 4′- amino-6′-aceto-phenoxyacetic acid.—The **disodium salt** separated on adding alcohol to its aqueous solution as pale brown, microscopic leaflets. The free acid was not obtained pure.

Analyses. Calc. for $C_{16}H_{14}O_7N_3AsNa_2.5H_2O$: H_2O, 15.77. Found: 16.36.

Calc. for $C_{16}H_{14}O_7N_3AsNa_2$: N, 8.73; As, 15.58. Found: N, 8.88; As, 15.50.

Diazo-2-methylbenzene-(4-arsonic acid) - *p* - amino-phenoxyacetic methyl ester.—*o*-Methylarsanilic acid was diazotized in the same way as arsanilic acid and the solution treated first with a large excess of saturated sodium acetate solution and then with an equivalent of *p*-amino-phenoxyacetic methyl ester hydrochloride[19] dissolved in water. The initially amorphous diazo-amino sodium salt crystallized on letting stand in the cold as spherules of drab, microscopic needles. The free **ester acid** forms sheaves, rosets, and plumes of minute, flat needles which decompose at 130–2°.

Analysis. Calc. for $C_{16}H_{18}O_7N_3As$: N, 9.93. Found: 10.02.

Diazo 2- methylbenzene- (4-arsonic acid) - *p*- amino-phenoxyacetic acid.—The ester, saponified as usual, yielded the **disodium salt** which separated from aqueous solution on adding alcohol as radiating masses of minute, brownish leaflets. The free acid was not obtained crystalline.

Analyses. Calc. for $C_{15}H_{14}O_6N_3AsNa_2.6.5H_2O$: H_2O, 20.53. Found: 20.80.

Calc. for $C_{15}H_{14}O_6N_3AsNa_2$: N, 9.27; As, 16.55. Found: N, 9.02; As, 16.64.

Diazo - 2- bromobenzene- (4-arsonic acid) - *p*- amino-phenoxyacetic methyl ester.—4.5 g. of 3-bromo-arsanilic acid[20] were suspended in 30 cc. of 2 *N* hydrochloric acid, diazotized with 1.1 g. of sodium nitrite, and the solution chilled and treated with 22.5 cc. of 2 *N* sodium hydroxide, then with an equal volume of satu-

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[18] *Loc. cit.*, p. 2215.

[19] *Ibid.*, p. 2196.

[20] Bertheim, *Ber.*, 43, 532 (1910).

rated sodium acetate solution and finally with a solution of 3.3 g. of the amino ester hydrochloride. The diazo-amino ester salt separated immediately as aggregates of pinkish, hair-like, microscopic needles. The ester acid forms spherules of brownish, microscopic needles which intumesce at 123–5°.

Analysis. Calc. for $C_{15}H_{15}O_6N_3BrAs$: N, 8.61. Found: 8.49.

Diazo - 2 - bromobenzene - (4-arsonic acid) - *p* - amino-phenoxyacetic acid.— The recrystallized disodium salt of the acid separates as drab-colored spherules of minute crystals.

Analyses. Calc. for $C_{14}H_{11}O_6N_3BrAsNa_2.4H_2O$: H_2O, 13.19. Found: 13.77.

Calc. for $C_{14}H_{11}O_6N_3BrAsNa_2$: N, 8.11; As, 14.47. Found: N, 7.88; As, 14.28.

The free arsonic acid is precipitated from 50% alcohol as drab microscopic crystals which intumesce slowly at about 130° with preliminary browning.

Analyses. Calc. for $C_{14}H_{13}O_6N_3BrAs$: N, 8.87; As, 15.80. Found: N, 8.89; As, 15.77.

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AROMATIC ARSENIC COMPOUNDS.

X. Azo Dyes Derived from Arsanilic Acid.

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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As stated in the previous paper, azo dyes containing arsenic were first described by Ehrlich and Bertheim[1] and later by Barrowcliff, Pyman, and Remfry,[2] and others. In our work with diazo-amino derivatives it was found that certain classes of aromatic amino compounds yielded amino-azo dyes at once when used as couplers with diazotized arsanilic acid, or gave diazo-amino compounds which rapidly rearranged into the dyes. This occurred with *N*-alkyl anthranilic acids, *m*-amino-phenoxyacetic acid and its substitution products, the amino-veratric acids, and the *o*-methyl and *o*-alkoxy derivatives of *N*-phenylglycine.

As far as we can find, no mention has been made of the use of *N*-alkylanthranilic acids for the synthesis of azo dyes. The addition of *N*-methyl anthranilic acid to the neutralized diazo solution results in the rapid formation first of the diazo-amino compound, which slowly rearranges to the dye on standing or changes over rapidly on acidification with acetic acid. A similar behavior was noted with *N*-ethyl- and *N*-*iso*-amylanthranilic acids.

Under the usual conditions of coupling in neutral or slightly acid solution, *m*-amino-phenoxyacetic acid yielded at once a dye in which we assume the azo group to enter the position *para* to the amino group. We believe that *m*-amino-phenoxyacetic acid functions here as a *m*-anisidine derivative as we have since noted the analogous experience of Reverdin, Rilliet and Vernei,[3] in which *p*-amino-azo dyes were prepared from *m*-phenetidine. Azo derivatives were also

[1] Ehrlich and Bertheim, *Ber.*, **40**, 3292 (1907).

[2] Barrowcliff, Pyman and Remfry, *J. Chem. Soc.*, **93**, 1893 (1908).

[3] C. *A.*, **13**, 312 (1919).

obtained by us from two groups of substitution products of *m*-ansidine and *m*-amino-phenoxyacetic acid, in one of which the position *para* to the amino group was free, and in the other of which this position was occupied by a substituent. In the former group were 4-methyl-5-amino-phenoxyacetic acid and 2-amino-3, 4-dimethoxy-benzoic acid, and in these cases the azo group presumably entered the free *para* position. In the second group were 2-methyl-5-amino-phenoxyacetic acid, 3-amino-6-methoxy-phenoxy acetic acid, 6-amino-*o*-veratric acid, 4-amino-6-methoxy-phenoxy-acetic acid, and amino-*o*-phenylene-di (oxyacetic acid); and in this group it is probable that the azo group enters the position *ortho* to the amino group. Thus German patent 230,592 describes the formation of *o*-amino-azo dyes from 2-methyl-5-amino-phenoxyacetic acid and from the analogous 2-methyl-5-amino-anisole, and our experience has confirmed the ready formation of azo dyes from such substances.

Finally, although phenylglycine yields principally a diazo-amino compound under the conditions described in the preceding paper, the *o*-methyl, *o*-methoxy, and *o*-ethoxyphenyl glycines at once form dyes. According to Mai,[4] however, when phenylglycine was coupled in a solution acid to congo red it yielded dyes with diazotized aniline and sulfanilic acid.

In most cases the reaction between the diazotized arsanilic acid and the coupler proceeded smoothly, with very little gas evolution. The isolation and purification of the resulting dyes often presented considerable difficulty, and much experimentation was necessary in order to discover the best conditions for the coupling and whether any dye were best isolated as the free acid, the monosodium salt, or the disodium salt. Hence the manipulative details are quite voluminous, and in order to save space it is deemed best to present the work in abstract form.

The monosodium salts of the dyes were generally very sparingly soluble in water, while the disodium salts dissolved easily. Even very dilute solutions of the latter gave immediate amorphous precipitates with salts of the heavier metals, while their behavior varied toward salts of the alkaline earths.

[4] Mai, *Ber.*, 35, 580 (1902).

EXPERIMENTAL.

To avoid constant repetition it may be stated here that the diazotization of arsanilic acid was carried out under the conditions given in the preceding paper in 2 mols. of normal hydrochloric acid with 5 *N* sodium nitrite. Since most of the amino acids were coupled under the conditions necessary for the formation of the monosodium salts of the resulting dyes, it will be understood unless otherwise stated that the diazo solution was first treated with one mol. of *N* sodium hydroxide solution, the solution thus containing diazobenzene arsonic acid and no free mineral acid. This solution was then slowly treated, with continued turbining and chilling, with a solution of the amino acid dissolved in one mol. of *N* sodium hydroxide solution, or treated with an additional mol. of alkali and then with a solution of the coupler hydrochloride in the case of basic couplers. Such a procedure will be indicated by the expression **B→A.** Cases in which the diazo solution was added to the coupler are designated by **A→B.** Variations from this procedure will be described as they occur.

The values given for water of crystallization were determined on the substances after they had come to equilibrium in the air.

(A) DYES DERIVED FROM ARSANILIC ACID AND METHOXY-NAPHTHYLAMINES.

1-Amino-2-methoxy-naphthalene-4′-azobenzene-4′-arsonic acid.—B→A, using β-methoxy-α-naphthylamine hydrochloride. The neamorphous acid was converted into the salt with just enough dil. sodium hydroxide solution. After salting out, dissolving in 50% alcohol, heating, and making just acid to congo red with hydrochloric acid, the arsonic acid slowly separated on rubbing and cooling as radiating aggregates of narrow, bluish-black, glistening, microscopic platelets, which did not melt below 285°. It dissolves in conc. sulfuric acid with a deep purple color and in dil. sodium hydroxide solution with a deep cherry-red tint.

Analyses. Calc. for $C_{17}H_{16}O_4N_3As.1.5H_2O$: H_2O, 6.3. Found: 5.9.

Calc. for $C_{17}H_{16}O_4N_3As$: N, 10.48; As, 18.68. Found: N, 10.42; As, 18.50.

1-Amino-4-methoxy-naphthalene-2-azobenzene-4′-arsonic acid.—Obtained like the preceding compound, the arsonic acid separated rapidly as dark brown, microcrystalline aggregates with a bronzy luster. It sintered and darkened when heated, softening to a tar at about 195° and intumescing at about 225°. It dissolves in conc. sulfuric acid with a purplish-red color, changing to brown-red, and becoming purplish again on dilution. In dil. sodium hydroxide solution the color is a deep cherry-red. It is soluble in acetic acid and alcohol.

Analyses. Calc. for $C_{17}H_{16}O_4N_3As.1.5\ H_2O$: H_2O, 6.3. Found: 6.0.
Calc. for $C_{17}H_{16}O_4N_3As$: N, 10.48; As, 18.68. Found: N, 10.31; As, 18.83.

(B) DYES OBTAINED FROM ARSANILIC ACID AND SUBSTITUTED AMINO-BENZOIC ACIDS.

4 - Methylamino - 5 - carboxy-phenylazo-benzene-4′-arsonic acid.—B (methylanthranilic acid) → A + equal volume saturated sodium acetate solution. The orange colored solution, which presumably contained the diazo-amino compound, deepened in color on standing, gradually depositing the orange **monosodium salt** in good yield. Recrystallized from boiling water containing a little sodium acetate, it forms radiating masses of short, brownish-yellow, microscopic needles which dissolve with difficulty in hot or cold water with an intense orange-yellow color.

Analyses. Calc. for $C_{14}H_{13}O_5N_3AsNa.2.5H_2O$: H_2O, 10.08. Found: 10.49.
Calc. for $C_{14}H_{13}O_5N_3AsNa$: N, 10.47. Found: 10.53.

Treated in a little water with one mol. of sodium hydroxide, then with alcohol to initial turbidity, the **disodium salt** separated in the cold. It forms bundles of flat, red, microscopic needles from 70% alcohol and dissolves readily in water with a deep orange color.

Analyses. Calc. for $C_{14}H_{12}O_5N_3AsNa_2.8.5H_2O$: H_2O, 26.57. Found: 26.63.
Calc. for $C_{14}H_{12}O_5N_3AsNa_2$: N, 9.93. Found: N, 9.83.

The monosodium salt, suspended in hot water, dissolved with a little sodium hydroxide, and treated with hydrochloric acid until just acid to congo red, gave the **free acid**, which when recrystallized from 50% alcohol forms brick-red, thin glistening microscopic needles and plates. It does not melt below 280° dissolves in conc. sulfuric acid with a deep orange color and gives a red solution in hot 10% hydrochloric acid, from which the hydrochloride separates on cooling as rosets of narrow, purplish-red plates with a steel-blue reflex. In dil. hydrochloric acid the dye reacts with sodium nitrite, giving a precipitate of pale salmon-colored, microscopic needles, presumably the nitroso compound.

Analyses. Calc. for $C_{14}H_{14}O_5N_3As$: N, 11.08; As, 19.78. Found: N, 11.24; As, 19.68.

4 - Ethylamino - 5 - carboxy-phenylazo-benzene-4′-arsonic acid.—B → A.—The crude monosodium salt was dissolved in hot water with a little sodium hydroxide and the solution just acidified to congo red, yielding the bright yellow arsonic acid. It separates from 85% alcohol as deep orange, glistening rectangular platelets which do not melt below 275°. It is more soluble in methyl alcohol or boiling amyl alcohol than in the other usual neutral solvents, and in its color reactions and chemical properties resembles the methylamino compound.

Analyses. Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69; As, 19.06. Found: N, 10.72 As, 19.11.

4 - iso-amyl-amino - 5 - carboxy-phenylazo-benzene-4′-arsonic acid.—B → A.—The crude **monosodium salt** when recrystallized from much water containing

a little sodium acetate forms orange-colored, microscopic leaflets which are very difficultly soluble in boiling water.

Analyses. Calc. for $C_{18}H_{21}O_5N_3AsNa.2H_2O$: H_2O, 7.31. Found: 6.59.

Calc. for $C_{18}H_{21}O_5N_3AsNa$: N, 9.19. Found: 9.18.

Recrystallized from acetic acid, the **free acid** forms orange-red, glistening, striated platelets which do not melt below 275°. It dissolves in boiling alcohol and gives a deep red-orange color with conc. sulfuric acid; in conc. hydrochloric acid it dissolves with a deep red color, the hydrochloride separating on rubbing as dark colored microscopic needles.

Analyses. Calc. for $C_{18}H_{22}O_5N_3As$: N, 9.66; As, 17.22. Found: N, 9.48; As 17.32.

4 - Amino - 2,3 - dimethoxy-5-carboxy-phenylazo-benzene-4′-arsonic acid.—B → A.—Using vicinal aminoveratric acid, the **monosodium salt** was obtained, separating as flat, brown-orange, microscopic needles from hot water containing a little sodium acetate.

Analyses. Calc. for $C_{15}H_{15}O_7N_3AsNa.2.5H_2O$: H_2O, 9.15. Found: 9.79.

Calc. for $C_{15}H_{15}O_7N_3AsNa$: N, 9.40; As, 16.77. Found: N, 9.76; As, 16.54.

The **free acid** separates from 85% alcohol as long, narrow, glistening, orange-red platelets which turn a deeper red when anhydrous, but do not melt below 275°. It is quite easily soluble in cold methyl alcohol and gives a deep orange-colored solution in conc. sulfuric acid, dissolving in boiling 10% hydrochloric acid with a dark red color, depositing the hydrochloride on cooling as brown platelets and flat needles.

Analyses. Calc. for $C_{15}H_{15}O_7N_3As.1.5H_2O$: H_2O, 5.98. Found: 6.43.

Calc. for $C_{15}H_{16}O_7N_3As$: As, 17.62. Found: As, 17.78.

2 - Amino - 4,5 - dimethoxy-3-carboxy-phenylazo-benzene-4′-arsonic acid.—(B→A.—Alkalinity sufficient for formation of the disodium salt.) 6-Amino-*o*-veratric acid[5] was used. After 24 hours about 20% of solid sodium acetate was added and then an excess of acetic acid, precipitating the **monosodium salt.** Recrystallized from water containing sodium acetate, cautiously adding acetic acid after cooling, the salt forms spherules of orange-red microcrystals which change to chocolate-brown when air-dry. It is very difficultly soluble in boiling water and dissolves on adding alkali with an orange color.

Analyses. Calc. $C_{15}H_{15}O_7N_3AsNa.1.5H_2O$: H_2O, 5.70. Found: 6.37.

Calc. for $C_{15}H_{15}O_7N_3AsNa$; N, 9.40; As, 16.77. Found: N, 9.21; As, 16.65.

The **free acid** separates from an alkaline solution of the salt in 50% alcohol on acidifying to congo red as glistening brown leaflets and microcrystalline aggregates with a purplish luster, containing about 1.5 molecules of water of crystallization and turning darker brown when dehydrated. It decomposes somewhat on

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[5] This acid was prepared by the following series of reactions, which will be dealt with in a later communication: *o*-vanillin → *o*-veratric aldehyde → 6-nitro-*o*-veratric aldehyde → 6-nitro-*o*-veratric acid → 6-amino-*o*-veratric acid.

heating, but does not melt up to 290°. It is difficultly soluble in the usual solvents, and unlike the monosodium salt, dissolves in dil. alkalies with a red color, only changing to orange at high dilution. It gives an intense red color in conc. sulfuric acid, and dissolves partially in conc. hydrochloric acid, the hydrochloride soon separating as dark orange-red microcrystalline spherules.

Analyses. Calc. for $C_{15}H_{16}O_7N_3As.1.5H_2O$: H_2O, 5.98. Found: 5.29.

Calc. for $C_{15}H_{16}O_7N_3As$: N, 9.89. Found: 9.69.

(c) DYES DERIVED FROM ARSANILIC ACID AND AROMATIC GLYCINES.

4-[Phenyl - (4′-arsonic acid) azo] - phenylglycine.—Although phenylglycine yielded the diazo-amino compound under the conditions given in the preceding paper, the dye was formed in the presence of an excess of mineral acid. The diazo solution from 4.4 g. of arsanilic acid was added, with stirring and chilling, to a solution of 3.1 g. of phenylglycine in 40 cc. of *N* hydrochloric acid diluted to 100 cc. with 0.5 *N* hydrochloric acid. The dye, which crystallized soon after deposition commenced, was filtered off, washed first with cold and then with hot water, and was finally cooled and washed with acetone. The yield was 5.3 g. A dilute solution in 50% alcohol was obtained with the aid of a little ammonia and acidified hot to congo red, the arsonic acid soon separating as orange-red aggregates of minute, lenticular platelets with a golden luster. It sinters, melts, and decomposes slowly at 170–5°, and dissolves in dil. alkali with a reddish-orange color. It dissolves in conc. sulfuric acid or 1 : 1 hydrochloric acid with a deep red color, the hydrochloride separating from the latter on rubbing as red microscopic needles with a purplish luster.

Analyses. Calc. for $C_{14}H_{14}O_5N_3As.0.5H_2O$: H_2O, 2.32. Found: 2.86.

Calc. for $C_{14}H_{14}O_5N_3As$: N, 11.07. Found: 11.43.

2 - Methyl - 4 - [phenyl - (4′-arsonic acid) azo] phenylglycine.—(A unneutralized → B in 2 mols. of alkali.) The dye salt separated on rubbing and was dissolved in 50% alcohol with the aid of sodium hydroxide and the red-orange solution then made just acid to congo red, the arsonic acid separating as red-brown needles, leaflets, and rhombic platelets. When rapidly heated to 155°, then slowly, the acid reddens and intumesces at 157°. It is quite soluble in the cold in methyl alcohol, and dissolves in conc. sulfuric acid with a deep red color. A solution in 1 : 1 hydrochloric acid deposits the hydrochloride on rubbing as minute, dark brown platelets with a purple luster.

Analyses. Calc. for $C_{15}H_{16}O_5N_3As.H_2O$: H_2O, 4.38. Found: 3.49.

Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69; As, 19.06. Found: N, 10.70; As, 19.08.

2 - Methoxy - 4 - [phenyl - (4′-arsonic acid) azo] phenylglycine.—(B in 1 mol. of alkali → A treated with one mol. of alkali and saturated with salt.) The monosodium salt gradually crystallized. It separates from dil. aqueous alcoholic alkali on adding acetic acid as very thin, nacreous, orange-red platelets containing about 2.5 molecules of water of crystallization when air-dry and dissolving in water

with a deep red color, changing to brown and depositing microcrystalline spherules of what appears to be a different hydrate; on adding sodium acetate or sodium hydroxide a clear, deep orange solution results.

Analyses. Calc. for $C_{15}H_{15}O_6N_3AsNa.2.5H_2O$: H_2O, 9.46. Found: 8.93.

Calc. for $C_{15}H_{15}O_6N_3AsNa$; As, 17.39. Found: 17.28.

The **acid** was obtained in the usual way as minute, glistening, steel-blue aggregates of platelets. When rapidly heated the air-dry acid melts and effervesces at about 160°, but if dried first it gradually reddens on heating and intumesces at 167° to a blood-red mass. It is quite soluble in the cold in methyl alcohol, easily in hot ethyl alcohol, and dissolves in conc. sulfuric acid with a deep purple color, in 1 : 1 hydrochloric acid with a deep red color, and in dil. solutions of carbonates or alkalies with an orange color.

Analyses. Calc. for $C_{15}H_{16}O_6N_3As.1.5H_2O$: H_2O, 6.19. Found: 6.11.

Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27. Found: N, 10.30.

2 - Ethoxy - 4 - [phenyl - (4′-arsonic acid) azo] phenylglycine.—On adding an equal volume of saturated sodium chloride solution to the turbined reaction mixture the **monosidium salt** separated as a paste, but crystallized on rubbing. From 50% alcohol it forms rosets of orange-brown, glistening platelets which dissolve in water with a brown-red color.

Analyses. Calc. for $C_{16}H_{17}O_6N_3AsNa.2.5H_2O$: H_2O, 9.19. Found: 8.68.

Calc. for $C_{16}H_{17}O_6N_3AsNa$: N, 9.44. Found: 9.63.

The **free acid** separates very incompletely as rosets and sheaves of flat, purplish brown, microscopic needles which decompose at 245–50° and are freely soluble in 50% alcohol and 50% acetic acid, especially on warming. The solution in conc. sulfuric acid is deep red, appearing purple in thin layers.

Analysis. Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 10.12.

α′ - [Phenyl - (4-arsonic acid) azo] - α - naphthylglycine.—The monosodium salt was precipitated from the reaction mixture with an equal volume of alcohol converted into the **disodium salt** by dissolving in one molecular equivalent of sodium hydroxide and adding alcohol until precipitation began, purifying by redissolving in warm 50% alcohol and treating with alchol until the concentration of solvent was about 85%, whereupon the salt crystallized. It dissolves readily in water with a purple color.

Analyses. Calc. for $C_{18}H_{14}O_5N_3AsNa_2$: N, 8.88; As, 15.85. Found: N, 9.00; As, 15.36.

The salt was dissolved in 25% alcohol, made just acid to congo red, and filtered at once from a trace of amorphous matter, after which the **free acid** soon crystallized. It melts and decomposes at about 275° with preliminary darkening and softening and dissolves in conc. sulfuric acid with a deep red color, appearing purple in thin layers.

Analyses. Calc. for $C_{18}H_{16}O_5N_3As.0.5H_2O$: H_2O, 2.1. Found: 3.0.

Calc. for $C_{18}H_{16}O_5N_3As$: N, 9.79. Found: 9.48.

(D) DYES DERIVED FROM ARSANILIC ACID AND AROMATIC *N*-METHYL SULFONIC ACIDS.

4 - [Phenyl - (4′ - arsonic acid) azo] - phenyl-aminomethyl sulfonic acid, $H_2O_3As C_6H_4N:NC_6H_4NHCH_2SO_3H$.—Methylene-aniline sodium bisulfite solution was added to neutralized A saturated with salt. The **disodium salt** of the dye soon began to crystallize on keeping in the cold, and the process was hastened by the addition of two-thirds of a volume of saturated sodium acetate solution. Recrystallized from 50% alcohol, the salt separates as delicate, orange-colored needles which dissolve readily in water.

Analyses. Calc. for $C_{13}H_{12}O_6N_3SAsNa_2.4.5H_2O$: H_2O, 15.01. Found: 15.09.

Calc. for $C_{13}H_{12}O_6N_3SAsNa_2$: N, 9.15; As, 16.32. Found: N, 9.63; As, 16.78.

The salt was dissolved in 25% alcohol and the solution acidified to congo red. The acid soon separated as sheaves of minute, flat, red needles with a purplish reflex. It dissolves with difficulty in cold water, readily on boiling, with a red color. When rapidly heated to 185°, then slowly, the anhydrous acid turns bright red, then sinters, and finally swells and melts partially at 187–9°. It dissolves in conc. sulfuric acid with a transient, deep orange color, quickly fading to yellow.

Analyses. Calc. for $C_{13}H_{14}O_6N_3SAs.2H_2O$: H_2O, 7.99. Found: 8.23.

Calc. for $C_{13}H_{14}O_6N_3SAs$: N, 10.12; As, 18.05. Found: N, 10.30; As, 18.25.

2 - Methoxy - 4 - [phenyl - (4′-arsonic acid) azo] - phenyl-aminomethyl sulfonic acid.—As in the preceding case, methylene-*o*-anisidine sodium bisulfite yielded a **disodium salt** which was dissolved in a small volume of water and treated cautiously with somewhat more than an equal volume of alcohol. On standing in the cold, the salt separated as orange-red, microscopic needles dissolving readily in water with a deep orange-red color.

Analyses. Calc. for $C_{14}H_{14}O_7N_3SAsNa_2.3H_2O$: H_2O, 9.95. Found: 9.96.

Calc. for $C_{14}H_{14}O_7N_3SAsNa_2$: As, 15.32. Found: 15.52.

In 50% alcohol, mineral acid causes the free **arsonic acid** to separate as lustrous flat, violet needles. The anhydrous acid reddens and softens above 155°, and melts with slow gas evolution at 158–60°, dissolving in conc. sulfuric acid with a deep purple color which changes to deep red on standing.

Analyses. Calc. for $C_{14}H_{16}O_7N_3SAs.1.5H_2O$: H_2O, 5.72. Found: 4.95.

Calc. for $C_{14}H_{16}O_7N_3SAs$: N, 9.44. Found: 9.28.

(E) DYES DERIVED FROM ARSANILIC ACID AND SUBSTITUTED PHENOXYACETIC ACIDS.

3 - Amino - 6 - [phenyl - (4′-arsonic acid) azo] - phenoxyacetic acid, $H_2O_3AsC_6H_4N:NC_6H_3(NH_2)OCH_2CO_2H$.—*m*-Amino-phenoxyacetic acid[6] yielded a deep red solution which was gently warmed and treated with alcohol, depositing the

[6] This Journal, 39, 2912 (1917).

orange microcrystalline **monosodium salt**, which was converted into the acid by dissolving in a considerable volume of dilute alcohol with the aid of sodium hydroxide and making the solution just acid to congo red. The **acid** separated as red, glistening needles. When anhydrous it blackens, but does not melt below 285° dissolving in conc. sulfuric acid with an orange-red color, in dil. alkalies with an orange color, and gives an orange-red color in 1:1 hydrochloric acid, the hydrochloride separating on rubbing as orange-brown microscopic crystals.

Analyses. Calc. for $C_{14}H_{14}O_6N_3As.1.5H_2O$: H_2O, 6.40. Found: 6.99.

Calc. for $C_{14}H_{14}O_6N_3As$: N, 10.63; As, 18.96. Found: N, 10.74; As, 18.75.

4 - Methyl - 3 - amino - 6 - [phenyl - (4′-arsonic acid) azo] - phenoxyacetic acid.—4-Methyl-5-amino-phenoxyacetic acid[7] gave the monosodium salt of the dye as a red, crystalline mass which was converted as usual into the acid, this separating as dark red aggregates of flat neeedles with a purple reflex. When rapidly heated to 240°, then slowly, the dye intumesces at 242–3°, with preliminary blackening. It gives an orange color in conc. sulfuric acid or dil. alkalies, and is difficultly soluble in conc. hydrochloric acid, changing on rubbing to an orange-red, microcrystalline hydrochloride.

Analyses. Calc. for $C_{15}H_{16}O_6N_3As.H_2O$: H_2O, 4.22. Found: 3.47.

Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27: As, 18.31. Found: N, 10.06; As, 18.51.

2 - Methyl - 5 - amino - 4 - [phenyl - (4′-arsonic acid) azo] - phenoxyacetic acid.—In the case of 2-methyl-5-amino-phenoxyacetic acid,[7] the deep red-brown solution of the monosodium salt was filtered, concentrated partially *in vacuo*, and allowed to stand in the cold. The resulting crystals were dissolved in boiling 85% alcohol containing acetic acid, and on cooling the free acid separated. Reprecipitated in 33% alcohol it forms flat, glistening, purplish-brown needles which dissolve readily in methyl alcohol, less easily in ethyl alcohol. When rapidly heated to 185°, then slowly, it intumesces at 187–8°. It dissolves in conc. sulfuric acid with a deep orange color and partially in conc. hydrochloric acid with a red shade, soon depositing the hydrochloride as dull red microcrystals.

Analyses. Calc. for $C_{15}H_{16}O_6N_3As.2H_2O$: H_2O, 8.09. Found: 7.07.

Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27. Found: 10.38.

3 - Amino - 6 - methoxy - 4 - [phenyl - (4′-arsonic acid) azo] - phenoxyacetic acid.—Precipitation of the dye acid obtained from 3-amino-6-methoxy-phenoxyacetic acid[8] was facilitated by the addition of acetic acid and the collected product was dissolved in dil. sodium hydroxide, filtered, and reprecipitated with an excess of acetic acid. Reprecipitated in 50% alcohol, the acid separates as flat, glistening, bronze needles and platelets. The air dry acid sinters, softens, and swells at 175–85°, while the anhydrous substance intumesces at 208–13° with preliminary sintering and softening. It dissolves appreciably in the cold in methyl or ethyl alcohol, readily on warming, and dissolves in dil. alkalies or car-

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[7] *Ibid*, 39, 2193 (1917).

[8] *Loc. cit.*, p. 2194.

bonates with an orange-red color. It gives a bright red color in conc. sulfuric acid and yields a deep purplish-red solution in 1:1 hydrochloric acid, almost immediately depositing the hydrochloride as dark brown, microscopic platelets.

Analyses. Calc. for $C_{15}H_{16}O_7N_3As.4.5H_2O$: H_2O, 16.02. Found: 15.47.

Calc. for $C_{15}H_{16}O_7N_3As$: N, 9.88. As, 17.64. Found: N, 9.89; As, 17.62.

4 - Amino - 6 - methoxy - 3 - [phenyl - (4′-arsonic acid) azo] - phenoxyacetic acid.—Using 4-amino-6-methoxy-phenoxyacetic acid,[9] the **monosodium salt** of the dye gradually separated best on concentrating to smaller bulk *in vacuo* and allowing to stand in the ice box. Recrystallized from dil. sodium acetate solution, it separates as chocolate colored, microscopic needles which are very sparingly soluble in water, but dissolve on adding carbonate or alkali with a dark red color.

Analyses. Calc. for $C_{15}H_{15}O_7N_3AsNa.6.5H_2O$: H_2O, 20.75. Found: 21.03.

Calc. for $C_{15}H_{15}O_7N_3AsNa$: As, 16.77. Found: 16.31.

The **free acid** can be precipitated in 50% alcohol as minute, glistening, maroon platelets. It does not melt below 280°, and dissolves in conc. sulfuric acid with a bright red color. A solution in hot 1 : 1 hydrochloric acid deposits the hydrochloride on cooling as dark red, flat needles.

Analyses. Calc. for $C_{15}H_{16}O_7N_3As.2H_2O$: H_2O, 7.81. Found: 7.89.

Calc. for $C_{15}H_{16}O_7N_3As$: N, 9.89. Found: 9.97.

4 - Amino - 5 - [phenyl - (4′ - arsonic acid) azo] - 1,2-bis-phenoxyacetic acid.—**A → B**, using 4-amino-1, 2-bis-phenoxyacetic acid.[10] After acidifying with acetic acid the **monosodium salt** of the dye separated as dark purplish-brown, microcrystalline spherules. It is very sparingly soluble in water, but dissolves with an orange-red color on adding carbonate or alkali.

Analyses. Calc. for $C_{16}H_{15}O_9N_3AsNa.4.5\ H_2O$: H_2O, 14.17. Found: 14.40.

Calc. for $C_{16}H_{15}O_9N_3AsNa$: N, 8.56. Found: 8.61.

When precipitated in much warm 50% alcohol, deposition of amorphous material is prevented and the **free acid** separates as dark purplish-brown microcrystals. Its repetition of this process was necessary before the acid was analytically pure. A dissolves in conc. sulfuric acid with an orange-red color, and when anhydrous, softens and sinters when heated, but does not melt below 280°.

Analyses. Calc. for $C_{16}H_{16}O_9N_3As.3H_2O$: H_2O, 10.33. Found: 10.58.

Calc. for $C_{16}H_{16}O_9N_3As$: N, 8.96. As, 15.97. Found: N, 9.07; As, 15.8

α - Amino - β-[phenyl - (4-arsonic acid) azo] - α′ - naphthoxyacetic acid.—α-Amino-α′-naphthoxyacetic acid[11] gave a deep purple, amorphous precipitate which was dissolved by adding 1 mol. of sodium hydroxide and the solution treated with several volumes of alcohol, causing deposition of the **disodium salt.** Recrystallized from 50% alcohol, it separates as dark brown, felted microscopic needles which dissolve readily in water with a dark purplish-red color.

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[9] *Loc. cit.*, p. 2212.

[10] *Ibid.*, p. 2216.

[11] *Ibid.*, p. 2217.

Analyses. Calc. for $C_{18}H_{14}O_6N_3AsNa_2.9H_2O$: H_2O, 24.89. Found: 25.25.

Calc. for $C_{18}H_{14}O_6N_3AsNa_2$: N, 8.59; As, 15.33. Found: N, 8.46; As, 15.71.

The **free acid** may be precipitated in much warm 50% alcohol as flat, dark red needles with a golden luster. It decomposes at about 285°, with preliminary darkening and softening. It dissolves in conc. sulfuric acid with a deep purplish-red color.

Analysis. Calc. for $C_{18}H_{16}O_6N_3As$: N, 9.44. Found: 9.47.

α-Amino-α′-[phenyl-(4-arsonic acid) azo]--β-naphthoxyacetic acid.— Sodium salt of α-amino-β-naphthoxyacetic acid → neutralized A. The deep purple solution was concentrated to small bulk *in vacuo* and treated with several volumes of alcohol, the **disodium salt** separating in the cold. Recrystallized from 50% alcohol it forms almost black aggregates of microscopic hairs with a greenish reflex and dissolving in water with a deep red color.

Analyses. Calc. for $C_{18}H_{14}O_6N_3AsNa_2.8.5H_2O$: H_2O, 23.84. Found: 23.64.

Calc. for $C_{18}H_{14}O_6N_3AsNa_2$: N, 8.59; As, 15.33. Found: N, 8.56; As, 15.81.

The **free acid**, when liberated in much 50% alcohol slowly separates as red-brown microcrystalline aggregates which do not melt below 285°.

Analyses. Calc. for $C_{18}H_{16}O_6N_3As.H_2O$: H_2O, 3.89. Found: 3.96.

Calc. for $C_{18}H_{16}O_6N_3As$: N, 9.44. Found: 9.49.

2-Hydroxy-5-[phenyl-(4′-arsonic acid) azo]-phenoxyacetic acid.— Neutral A → alkaline *o*-hydroxy-phenoxyacetic acid solution. The neutralized solution was concentrated to small bulk *in vacuo*, and the dye precipitated by adding strong sodium hydroxide solution. Dissolved in hot 50% alcohol and treated with an excess of acetic acid, the **monosodium salt** separated as yellow, indefinitely crystalline, microscopic globules.

Analyses. Calc. for $C_{14}H_{12}O_7N_2AsNa$: N, 6.70; As, 17.93. Found: N, 6.42; As, 18.33.

The **free arsonic acid** separated from a solution in dil. sodium hydroxide as an emulsion which soon crystallized. Dissolved in boiling 85% alcohol, and the filtrate diluted with water, the acid separated as brown, microscopic platelets which do not melt below 280°. It dissolves in boiling alcohol, and gives a bright orange solution in dil. sodium hydroxide. The color in conc. sulfuric acid is a deep red-orange.

Analyses. Calc. for $C_{14}H_{13}O_7N_2As.0.5H_2O$: H_2O, 2.2. Found: 2.4.

Calc. for $C_{14}H_{13}O_7N_2As$: N, 7.07; As, 18.92. Found: N, 6.99; As, 18.61.

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ON THE STRUCTURE OF THYMUS NUCLEIC ACID AND ON ITS POSSIBLE BEARING ON THE STRUCTURE OF PLANT NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 1, 1921.)

The polynucleotide structure of both yeast and animal nucleic acids is generally accepted; the question of the mode of linking of individual nucleotides continues to be the subject of discussion and of disagreement between individual workers. Thannhauser, Jones, and the present writer, have each put forth a different theory of the structure of the plant nucleic acid. Each author has criticized the theories of the other two. As a result of these criticisms, Thannhauser[1] has modified his theory and incorporated in his formulation the views of Jones. Thus the criticism of the present author of the theory of Jones applies also to that of Thannhauser. It is peculiar that Jones, in the latest edition of his monograph, in discussing the theories of the structure of yeast nucleic acid does not at all refer to the theory of the present writer.

The point of contention is the following: Are the nucleotides united one to another in an ether linking through their carbohydrates, or in ester form, the phosphoric acid of one combining with the carbohydrates of the other? Jones, and with him Thannhauser, accepts the ether linking, the present writer the ester linking. Since the original evidence on which Jones and Thannhauser have based their theories was proved an experimental error, Jones[2] has furnished two new experiments in support of his theory. The first is the following:

The curve expressing the rate of hydrolysis of yeast nucleic acid is identical with that of a mixture of the four nucleotides. Accepting the experiment as correct, what does it demonstrate? It proves that

[1] Thannhauser, S. J., and Sachs, P., *Z. physiol. Chem.*, 1920–21, cii, 187.

[2] Jones, W., *Am. J. Physiol.*, 1920, lii, 193, 203.

the union between individual nucleotides is more labile than that between the phosphoric acid and the carbohydrate in each nucleotide. It is then self-evident that the first step in the hydrolysis of the nucleic acid molecule is the formation of four nucleotides. The further progress of hydrolysis of the nucleic acid is the same as of four nucleotides. This ready formation of four nucleotides as the initial phase is hard to reconcile with the assumption of Jones of an ether linking between individual nucleotides. On the other hand, this observation is consistent with the theory of the present writer. Truly, the cleavage of nucleic acid into mononucleotides should have been impossible if it were otherwise. The view of Levene is substantiated by the observations of Levene, Meyer, and Yamagawa,[3] who found that the rate of hydrolysis of phosphoric acid is practically identical whether 1–2–acetone–3- or 5–phosphoric acid–6–benzoyl glucose, or 1–2–acetone–3- or 5–phosphoric acid glucose are hydrolyzed.

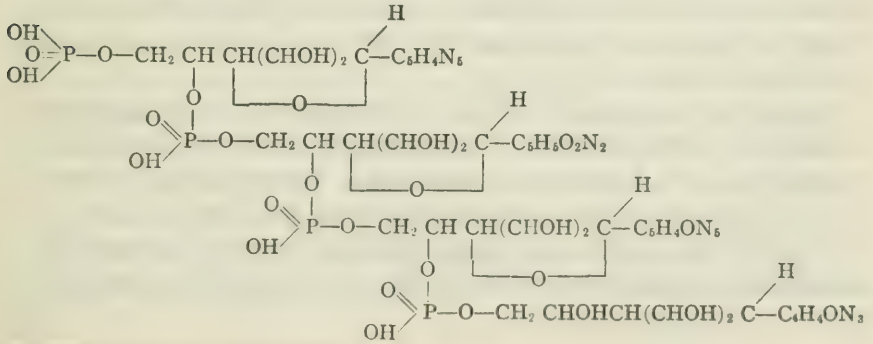
The second experimental proof of Jones' theory is the following: By a pancreas enzyme, yeast nucleic acid was cleaved to its nucleotides. At the starting point of the experiment the hydrogen ion concentration of the reacting mixture was brought to pH = 6.4, and at the end of the experiment there was no apparent change of the color of the indicator added to the original solution. Hence the author concludes that no acid radicles could be liberated as the result of the hydrolysis. The reasoning is not correct. According to either theory, nucleic acid is a polyphosphoric acid and when brought to a pH = 6.4 it possesses considerable buffer effect. Furthermore, each nucleotide is a comparatively weak acid and when liberated does not affect the hydrogen ion concentration of the buffer very markedly. Since the dissociation constant of the nucleotides has not been measured, it is not possible to express the reaction in quantitative terms. Experimentally, however, we convinced ourselves that when a solution of guanosinphosphoric acid is brought to a pH = 6.4 it stands the addition of an equal volume of a solution of free guanosinphosphoric acid of the same concentration before any change of color of the indicator can be noticed. Taking further into consideration the fact that a solution of nucleic acid is not perfectly colorless, that an extract of the pancreas always contains a considerable quantity of phosphates and

[3] Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xlii, 323. Levene P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlviii, 233.

also is not colorless, one easily realizes that the argument of Jones carries but little weight.

Thus, to the mind of the present writer, there exists neither experimental nor theoretical evidence in favor of the theory of the ether linking between individual nucleotides.

On the other hand, the theory of the present writer brings the structure of the yeast nucleic acid in harmony with that of thymus nucleic acid. Levene and Jacobs[4] have described hexothymidindiphosphoric acid and hexocytidindiphosphoric acid obtained on hydrolysis of thymus nucleic acid. They have also isolated hexothymidinmonophosphoric and hexocytidinmonophosphoric acids, and a substance which seemed to them to be a dinucleotide of hexothymidin and hexocytidin. In view of the experience on yeast nucleic acid, it seemed urgent to reinvestigate the question of the dinucleotide. For this purpose larger quantities of the material were required and hence it was attempted to simplify the method of its preparation. The complicated process employed in the older work was abandoned. From the hydrolyzed material the diphosphonucleotides were removed as barium or calcium salt on heating; from the mother liquor of these it was hoped to isolate the monophosphoric nucleotides and the hypothetical dinucleotide. However, under these conditions only the diphosphoric nucleotides could be isolated. It is possible that the monophosphoric acid nucleotides are secondary products, and that the hypothetical dinucleotide is only a mixture of mononucleotides. Thus for the present, until the existence of the dinucleotide is definitely proved, the structure of the thymus nucleic acid should be expressed in analogy with the yeast nucleic acid, namely as follows:



[4] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 411.

Incidentally the barium salt of the hexothymidindiphosphoric acid was obtained in crystalline form.

For the final solution of the problem of the structure of thymus nucleic acid larger quantities of the material are needed. Since the manufacture of this nucleic acid in Europe has been discontinued, it will have to be prepared with our laboratory facilities. Because of this, the progress of the work will be delayed, but work is now in progress.

EXPERIMENTAL.

Commercial animal nucleic acid (Merck) was hydrolyzed in 100 gm. lots. This amount of the acid was heated on flame with reflux condenser for 2 hours with 1,000 cc. of 2 per cent sulfuric acid. The sulfuric acid and the free phosphoric acid are removed by a slight excess of barium hydroxide solution. The excess of this reagent was then removed quantitatively and the solution concentrated under diminished pressure at room temperature to a volume of 300 cc. The nucleotides were then precipitated with a 25 per cent solution of neutral lead acetate. The precipitate was washed repeatedly with cold water and then suspended in water, decomposed by means of hydrogen sulfide gas. The filtrate from the lead sulfide was again concentrated under diminished pressure at room temperature to a volume of 300 cc. This was again neutralized with barium hydroxide, filtered from the slight trace of barium phosphate, and brought to a boil over a free flame. A flocculent precipitate soon appears, which on prolonged boiling assumes a granular character. The filtrate from this precipitate was then concentrated to small volume and again heated as before; generally a second precipitate formed. The material which formed on boiling (Precipitate I) had the composition of the diphosphonucleotides.

The mother liquor from the diphosphonucleotides was precipitated by alcohol (Precipitate II). This precipitate had the elementary composition of monophosphonucleotides. However, when freed from barium and purified through conversion into lead salt and reconversion with barium salt again, there is formed a barium salt insoluble in boiling water. Thus finally, practically all is converted into the diphosphonucleotides.

There seems to be a discrepancy between the present result and that obtained by Levene and Jacobs. Two alternative explanations may be given to the discrepancy; either the monophosphonucleotides found previously by Levene and Mandel and by Levene and Jacobs are products of further decomposition of the diphosphonucleotides formed in the course of further manipulation, or the monophosphonucleotides are missed in the present procedure.

Composition of Crude Barium Salts.

No. of sample.	P	N
358 20/21	8.82	5.36
359 20/21	8.27	5.14
361 20/21	7.74	5.89
366 20/21	7.39	5.62
370 20/21	8.27	5.31
362 20/21	5.66	5.96
474 20/21	5.15	6.11

Samples 362 and 474 were combined, freed from barium, converted into lead salt, and this reconverted into barium salt. The greater part settled out on boiling and had the following composition.

No. of sample.	P	N
408 20/21	7.17	5.50

For further purification and for the separation of individual diphosphonucleotides the older procedure was modified. The barium salts were converted into lead salts and these into brucine salts. The brucine salts were fractionated by recrystallization from 35 per cent alcohol until the more insoluble fraction had the elementary composition of the hexothymidindiphosphoric brucine salt. The combined mother liquors were then concentrated and allowed to stand until a crystalline deposit formed. This was again refractionated. After two refractionations the more soluble brucine salt on conversion into the barium salt analyzed for the hexocytidindiphosphoric acid barium salt.

Analysis of the Brucine Salts.

The most insoluble fraction of the brucine salt analyzed as follows:

0.1001 gm. of the substance on combustion gave 0.1999 gm. of CO_2 and 0.0606 gm. of H_2O.

0.2000 gm. of the substance gave 11.4 cc. of nitrogen gas at $T = 21°C.$ and $P = 751$ mm.

0.3000 gm. of the substance gave on fusion 0.288 gm. of $Mg_2P_2O_7$.

$C_{11}H_{18}N_2P_2O_{13}C_{92}H_{104}N_8O_{16}$	Calculated.	C 54.29, H 6.64, N 6.15, P 2.73.
$+14H_2O$.	Found.	C 54.45, H 6.77, N 6.54, P 2.67.

The most soluble fraction analyzed as follows:

0.1004 gm. of the substance gave on combustion 0.1990 gm. of CO_2 and 0.0591 gm. of H_2O.

0.2000 gm. of the substance gave 12.0 cc. of nitrogen gas at $T = 23°C.$ and $P = 752$ mm.

0.3000 gm. of the substance gave 0.0287 gm. of $Mg_2P_2O_7$.

$C_{10}H_{17}N_3P_2O_{12}{\cdot}C_{92}H_{104}N_8O_{16}$	Calculated.	C 54.10, H 6.64, N 6.81, P 2.75.
$+14H_2O$.	Found.	C 54.05, H 6.58, N 6.85, P 2.67.

This material was then converted into the barium salt. It must be remarked, however, that the elementary compositions of the brucine salts of the above two nucleotides do not differ sufficiently one from another to permit identification of the nucleotide on the basis of the analysis of the brucine salts. Often, samples, which on the basis of the analysis of the brucine salt seemed to be the cytidin nucleotide, proved on conversion into the barium salt to be the thymidin nucleotide.

Conversion of Brucine Salts into Barium Salts.

For this purpose the brucine salts were dissolved in 35 per cent alcohol, an excess of ammonia water added, and the product allowed to stand in the refrigerator. The brucine was then removed by filtration, the filtrate was again concentrated and allowed to stand to permit further crystallization of brucine. The operation was repeated as long as brucine crystallized out. From the final solution the nucleotides were precipitated by a 25 per cent solution of neutral lead acetate. The lead salt was washed repeatedly with water, filtered, suspended in water, and freed from lead by means of hydrogen

sulfide. The filtrate from lead acetate is concentrated under diminished pressure at room temperature and then converted into the barium salt.

The hexothymidin salt was then converted into the crystalline form.

Preparation of Crystalline Hexothymidindiphosphoric Barium Salt.

9 gm. of the barium salt obtained from the brucine salt were taken up in 500 cc. of water and shaken for 1 hour. Part remained insoluble. All was allowed to stand over night when a crystalline deposit was found covering the undissolved amorphous material. The mixed deposit was then taken in 1.5 liters of water at 30°C. and shaken for 1 hour in a shaking machine. The insoluble part was removed by filtration and the filtrate concentrated under diminished pressure at room temperature to a volume of 350 cc. After several hours of standing there appeared a crystalline deposit consisting of long needles grouped into star-shaped aggregates.

The composition of the substance was the following.

0.1050 gm. of the substance gave 0.0700 gm. of CO_2 and 0.0182 gm. of H_2O.

0.1719 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 4.97 cc. of 0.1 N acid.

0.2579 gm. of the substance gave on fusion 0.0830 gm. of $Mg_2P_2O_7$.

$C_{10}H_{13}N_3P_2O_{12}Ba_2$.	Calculated.	C 18.37, H 1.97, N 3.89, P 8.62.
	Found.	C 18.11, H 1.93, N 4.04, P 8.97.

Barium Salt of Hexocytidindiphosphoric Acid.

This was prepared from the brucine salt in the same manner as the thymidin salt. As yet it has not been converted into the crystalline form.

It analyzed as follows:

0.1052 gm. of the substance gave on combustion 0.0658 gm. of CO_2 and 0.0260 gm. of H_2O.

0.1870 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.21 cc. of 0.1 N acid.

0.2805 gm. of the substance gave 0.0946 gm. of $Mg_2P_2O_7$.

$C_{10}H_{13}N_3P_2O_{12}Ba_2$.	Calculated.	C 17.05, H 1.86, N 5.97, P 8.81.
	Found.	C 17.05, H 2.12, N 6.17, P 9.40.

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PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 7, 1921.)

In recent years a number of publications have appeared dealing with the problem of animal nucleic acids. The greatest part of the work was done by Steudel and by Feulgen, and more recently there appeared a publication by Thannhauser. The work from the Berlin laboratory was done during the years of the war and became accessible to us in original form only recently.

Some of the work of Feulgen[1] and the recent publication of Thannhauser deal with the problem of the mode of linking of the nucleotides in the tetranucleotide. Our tentative view on this part of the problem is expressed in another article, and we shall not refer to it here.

Other articles deal with the problem of the occurrence of animal nucleic acids of two new types. The acids of one type contain only three instead of the four nucleotides, thus the new type of acids from this view-point are not tetra- but trinucleotides. It was claimed to have been isolated from the pancreas gland. On the other hand Feulgen claimed to have isolated a more complex nucleic acid containing besides the hexose nucleotides also a pentose nucleotide; namely, guanylic acid. We shall refer to these acids as mixed nucleic acids. The evidence adduced by Feulgen and later by Hammarsten[2] in favor of the existence of mixed nucleic acids did not seem to us convincing.

[1] Feulgen, R., *Z. physiol. Chem.*, 1913, lxxxviii, 370; 1917, c, 241; 1917–18, ci, 288. Feulgen, R., and Landmann, G., *Z. physiol. Chem.*, 1918, cii, 262. Feulgen, R., *Z. Physiol. Chem.*, 1919–20, cviii, 147.

[2] Hammarsten, E., *Z. physiol. Chem.*, 1920, cix, 141.

Also the evidence in favor of the existence of a trinucleotide in the pancreas seemed to us to require further corroboration.

Recently we were forced to resume the preparation of animal nucleic acid required for our work since the technical production of the substance in Europe has been discontinued. This necessity gave us the occasion to repeat some of our older work, with the result that we have improved somewhat our older method of preparation of the nucleic acids and the method of analysis of the purine bases in nucleic acids. In the main the method of preparation is the same as previously used by us; the new details are given in the experimental part.

For the analysis of the purine bases it was found convenient to employ alcoholysis instead of hydrolysis.

If nucleic acid is suspended in methyl alcohol and hydrochloric acid gas is passed through the solution the nucleic acid is rapidly dissolved and soon the purine bases begin to separate in the form of their hydrochloric acid salts. The separation is practically completed in 2 hours. The heat developed by the absorption of the gas is sufficient to bring about the desired cleavage.

The bases are colored very little and are readily isolated for analysis. The acids were obtained from three organs: spleen, pancreas, and liver. These three organs were selected for the reason that they are all known to contain guanylic acid together with the animal nucleic acid.

From the spleen nucleic acid, the present writer obtained a nucleic acid[3,4] which contained the bases in the following proportions; adenin 8.17 per cent, guanin 9.15 per cent, cytidin 7.0 per cent, and thymin 8.0 per cent. The theory for a tetranucleotide requires, respectively, 9.56, 10.72, 7.86, and 8.93 per cent. In a recent paper, Steudel[5] writes that the presence of a true nucleic acid in the spleen is still in need of proof. His method of proving it is not more rigorous than the one employed by the previous writer.

The substances now prepared had the following composition:

[3] Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 370.

[4] Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 215.

[5] Steudel, H., *Z. physiol. Chem.*, 1921, cxiv, 255.

	C	H	N	P
Spleen nucleic acid	36.15	4.07	15.52	9.71
Pancreas " "	36.20	4.76	15.26	9.58
" " "	35.07	5.43	14.95	9.59
Liver " "	36.75	4.65	11.65	10.60
" " "	37.08	4.96	11.51	10.31
Theory for a hexosetetranucleotide	36.30	4.19	14.79	8.73

None of the samples showed the presence of pentose and all, even the samples from the liver, contained the two purine bases. The yield of the bases was smaller, however, from the pancreas and liver nucleic acids. They were as follows:

From:	Adenin picrate.	Crude guanin.
	gm.	*gm.*
Spleen nucleic acid	16.0	10.0
Pancreas " "	9.0	7.5
Liver " "	8.0	7.6

It is obvious that the elementary composition of amorphous substances of the nature of nucleic acids, which are never free from impurities, is of comparatively little service for the purpose of forming theories of molecular structure. However, great deviations from the theory, such as are observed on liver nucleic acid, require an explanation. Also, the fact that the pancreas nucleic acid, which in its elementary composition does not differ much from the spleen nucleic acid and yet furnishes on hydrolysis less purine bases than the other, needs further explanation. Work on these problems is now in progress.

EXPERIMENTAL.

Preparation of Nucleic Acids.—This is uniform for all three of the tissues, and the details of the procedure are as follows:

2,500 gm. of minced fresh tissue (previously freed from fat) are taken up in 3,000 cc. of water. 300 gm. of sodium chloride are added and all is kept boiling (with a steam coil) for 4 hours. Then 80 gm. of

sodium acetate and 60 cc. of a 33 per cent solution of sodium hydroxide are added, and the mixture is allowed to stand over night. The mixture is then neutralized with acetic acid and treated with picric as long as a precipitate forms. To the filtrate of this mixture hydrochloric acid is added until it turns slightly opalescent and the nucleic acid is precipitated with a 10 per cent solution of copper chloride. The copper salt of the nucleic acid is filtered and converted into the free acid by treatment with a 5 per cent solution of hydrochloric acid. The treatment is repeated once. The resulting free nucleic acid is redissolved in a 5 per cent solution of sodium hydroxide, the solution is made acid with acetic acid and precipitated with 95 per cent alcohol, containing 4 per cent of hydrochloric acid. The precipitate is then washed with 95 per cent alcohol until the washing no longer shows the presence of chlorine ions. The nucleic acid is then washed with absolute alcohol and ether, and dried. The substance so obtained does not show the presence of even traces of biuret-giving substances, and gives a negative test with orcin.

Alcoholysis.

10 gm. of nucleic acid are suspended in 200 cc. of dry methyl alcohol and hydrochloric acid gas is passed in a lively stream. After 2 hours the reaction is interrupted and the flask is allowed to stand over night. Guanin and adenin are separated in the usual way.

Guanin was purified by conversion into the sulfate, which again was converted into the free base. Adenin picrate for purification was twice recrystallized out of 10 per cent acetic acid and then dissolved in water by the addition of the required quantity of ammonia and precipitated by means of acetic acid.

Analysis of Nucleic Acids.

Spleen Nucleic Acid.

0.9880 gm. of the substance gave on combustion 0.1310 gm. of CO_2 and 0.0360 gm. of H_2O.

0.0884 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 9.8 cc. of 0.1 N acid.

0.1768 gm. of the substance gave 0.0616 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{40}H_{59}N_{15}P_4O_{32}$. *per cent*	Found. *per cent*
C	36.30	36.15
H	4.19	4.07
N	14.79	15.52
P	8.83	9.71

Bases.—10.0 gm. of this material yield 1.6 gm. of adenin picrate and 1.0 gm. of guanin. The guanin was analyzed as the free base.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required 66.30 cc. of 0.1 N acid for neutralization.

	Calculated for $C_5H_5N_5O$. *per cent*	Found. *per cent*
N	46.53	46.41

The adenin picrate analyzed as follows:

0.0946 gm. of the dry substance employed for Kjeldahl nitrogen estimation (reduced with zinc metal) required for neutralization 21 cc. of 0.1 N acid.

	Calculated for $C_5H_5N_5.C_6H_2(NO_2)_3OH$. *per cent*	Found. *per cent*
N	30.71	31.06

Pancreas Nucleic Acid.

A. 0.1056 gm. of the dry substance on combustion gave 0.1358 gm. of CO_2 and 0.0572 gm. of H_2O.

B. 0.1080 gm. of the dry substance of a second sample gave 0.1434 gm. of CO_2 and 0.0460 gm. of H_2O.

A. 0.1818 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 19.40 cc. of 0.1 N acid.

B. 0.1780 gm. of another sample required for neutralization 19.40 cc. of 0.1 N acid.

A. 0.2726 gm. of the substance gave 0.0938 gm. of $Mg_2P_2O_7$.

B. 0.2670 gm. of another sample gave 0.0918 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{40}H_{59}N_{15}P_4O_{32}$. *per cent*	Found. *per cent*	
		A	B
C	36.30	35.07	36.02
H	4.19	5.43	4.76
N	14.79	14.95	15.26
P	8.73	9.59	9.58

Bases.—The substance was alcoholyzed in 10.0 gm. lots. The average yield was 0.900 gm. of adenin picrate and 0.750 gm. of crude guanin. Guanin was analyzed as the free base and gave the following values.

0.1066 gm. of the substance gave 0.1566 gm. of CO_2 and 0.0310 gm. of H_2O.

0.0996 gm. of the substance gave 39.4 gm. nitrogen gas at $T = 26°$ and $P = 756$ mm.

	Calculated. for $C_5H_5N_5O$.	Found.
	per cent	*per cent*
C	39.73	39.05
H	3.30	3.26
N	46.60	46.62

Adenin picrate analyzed as follows:

0.0935 gm. of substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.25 cc. of 0.1 N acid.

	Calculated for $C_5H_5N \cdot C_6H_2(NO_2)_3OH$.	Found.
	per cent	*per cent*
N	30.71	30.32

Liver Nucleic Acid.

A. 0.9720 gm. of the dry substance gave 0.1330 gm. of CO_2 and 0.0434 gm. of H_2O.

B. 0.1090 gm. of another sample gave 0.1390 gm. of CO_2 and 0.0566 gm. of H_2O.

A. 0.0900 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 7.40 cc. of 0.1 N acid.

B. 0.1816 gm. of another sample required for neutralization 13.60 cc. of 0.1 N acid.

A. 0.1800 gm. of the dry substance gave 0.0666 gm. of $Mg_2P_2O_7$.

B. 0.2724 gm. of another sample gave 0.1108 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{43}H_{69}N_{15}P_4O_{32}$.	Found.	
	per cent	*per cent*	
		A	B
C	36.30	37.08	34.79
H	4.19	4.96	5.81
N	14.79	11.51	10.48
P	8.73	10.31	11.33

Bases.—One lot of 50.0 gm. was treated in methyl alcohol and hydrochloric acid gas as described above. This yield was 3.8 gm. of crude guanin and 4.0 gm. of crude adenin picrate.

Guanin was identified as the free base and analyzed as follows:

0.0997 gm. of the base gave on combustion 0.1566 gm. of CO_2 and 0.0338 gm. of H_2O.

	Calculated for $C_5H_5N_5O$.	Found.
	per cent	*per cent*
C	39.73	39.55
H	3.30	3.47

Adenin was identified as picrate and analyzed as follows:

0.0940 gm. of the substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.50 cc. of 0.1 N acid.

	Calculated for $C_5H_5N_5 \cdot C_6H_2(NO_2)_3OK$.	Found.
	per cent	*per cent*
N	30.71	30.53

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THE LIVER LECITHIN.

By P. A. LEVENE and H. S. SIMMS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The older work on the unsaturated lipoids of the liver has been reviewed in the paper of Levene and Ingvaldsen. In the same paper a new analysis of the liver lecithin was reported. The new facts reported in that paper related principally to the nature of the fatty acids. In the course of the work reported by Levene and Ingvaldsen[1] two fatty acids were found, one saturated, and the other unsaturated, which analyzed for a polyunsaturated arachidic acid. Since an acid of that structure has never before been described in connection with lecithin, further corroboration of the finding seemed desirable. Furthermore, in the course of the present year it was shown by Levene and Rolf[2] that the egg lecithin and that of the brain contained two saturated fatty acids. This result was obtained by means of fractional distillation of the esters of the combined saturated acids. In light of this observation it became necessary to reinvestigate all lecithins in regard to the number of fatty acid radicles, saturated and unsaturated, present in their molecule. In regard to the liver lecithin it was now found that it contained two saturated and two unsaturated acids.[3]

The saturated acids are palmitic and stearic. They were isolated and identified by the same procedure as described by Levene and Rolf.

The unsaturated acids are: one, unsaturated stearic, and the other, unsaturated arachidic. On reduction one is converted into stearic and the other into arachidic. The exact degree of unsaturation of either one of the two acids is as yet not known. There are, however, indications that one (arachidic) may be tetra-unsaturated. On the

[1] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

[2] Levene, P. A., and Rolf, Ida P., *J. Biol. Chem.*, 1921, xlvi, 193, 353.

[3] Evidence has recently been obtained in this laboratory that egg lecithin also contains the two unsaturated acids.

addition of bromine a substance was obtained which analyzed for an octobromide of arachidic acid. However, it will require a larger quantity of material to establish the degree of unsaturation of each of the two acids with certainty. The presence of several acids in the liver lecithin again emphasizes the question of the existence of more than one lecithin.

It was attempted to answer this question by the molecular weight estimation of the hydrolecithin. The hydrolecithin from the liver lecithin has been prepared essentially according to Paal's procedure.

The molecular weight of the substance was found 810 and 700 (in two estimations). The theory of a monophosphatide requires 809, that of a diphosphatide 1600. Consequently, liver lecithin consists of a mixture of monolecithins.

In the course of the present work the process of preparation of pure free lecithin from its cadmium chloride salt has been improved so that analytically pure substance is prepared in good yield; namely, about 50 gm. of free lecithin from 100 gm. of the cadmium chloride salt.

The procedure in the main is as follows: The salt of lecithin is dissolved in chloroform and this solution is transferred into a solution of dry ammonia gas in dry methyl alcohol. The resulting lecithin is purified from the slight quantity of impurities by the acetic acid process developed by Levene and Ingvaldsen. The details of the procedure are given in the experimental part.

EXPERIMENTAL.

I. Preparation of Pure Lecithin.

Various attempts to produce pure liver lecithin without converting it into the cadmium chloride salt met with little success. The following method proved to be the easiest and most efficient.

The liver, in 100 pound lots, is minced, dried, and extracted, first with acetone, second with ether, and last with alcohol. These extracts are treated separately as follows:

Acetone Extract.—This is allowed to stand at 0°C. over night. A precipitate of fat is deposited, which is removed by filtration. The filtrate is concentrated (if necessary) and the lecithin precipitated by adding a saturated solution of cadmium chloride in alcohol until no further precipitate is formed.

The residue from the above filtration is suspended in alcohol and warmed until the fat is melted. The mixture is then cooled over night. The fat precipitated on standing is again filtered from the alcoholic solution and again treated with alcohol as before. This extraction is repeated until the mother liquor no longer gives with cadmium chloride a precipitate of lecithin cadmium salt. The latter is recognized by the fact that on dissolving in a small amount of moist ether it is again precipitated by the addition of an excess of acetone. From three to seven extractions may be required. The alcoholic mother liquors are then precipitated with cadmium chloride.

Ether Extract.—This is concentrated to a small volume and allowed to stand at 0°C., when a precipitate consisting of fat and cerebrosides is formed. The precipitate is extracted with ether. The ethereal extract is added to the original filtrate and cooled once more to permit the separation of the cerebrosides which the solution may still contain. After filtering, the combined mother liquors are concentrated and treated with alcohol to separate the lecithin from cephalin. The alcoholic liquors are then treated with cadmium chloride.

Alcoholic Extract.—This is likewise concentrated and cooled to remove cerebrosides, the mother liquor being decanted if possible, otherwise filtered, or centrifuged if necessary.

The cerebrosides are again extracted with warm ether. The extract is cooled and centrifuged. The alcoholic and ethereal liquors are then treated with cadmium chloride.

Treatment of Cadmium Chloride Salts.

It is necessary to allow the cadmium chloride precipitate of lecithin to stand at least half an hour until it is sufficiently coagulated to permit filtration. The filtered material, which is not quite dry, is transferred to a large beaker or precipitating jar and stirred up with a large volume of cold acetone. If the acetone liquor turns dark from dissolved material the suspension is allowed to settle, the liquor decanted off, and more cold acetone is added. Finally the material is filtered by suction.

This material is purified in two steps: the one is the "ether crystallization," the other is the "toluene-ether" process.[1] It is a matter

of judgment as to which shall be used first and the number of times which each should be repeated. The aim is to obtain a white granular material which filters quickly.

The ether crystallization consists in dissolving the cadmium chloride salt in warm ether, water being added, a few drops at a time, until the suspended material goes into solution. An excess of water hinders the solution of the larger particles. The solution is allowed to stand over night, or longer, at 0°C. The substance should separate in a granular form, easily filterable by suction. If it forms a pasty solution not easily filtered, time and material will be wasted in attempting a filtration. Another precipitation with acetone should remove impurities which interfere with the process.

This purification removes not only the fats and oils but also takes out most of the cephalin present. Since the cadmium chloride salt is itself slightly soluble in cold ether, some of the material may be lost in purification. Hence the following precautions are necessary. 1. Excess of ether is to be avoided. With very impure material it is more advisable to repeat the purification several times with small quantities of solvent than to use a large excess at one time. The amount of ether filtered off should not be more than twice the volume of the residue. 2. The filtered material should *not* be washed with ether, but should be filtered as quickly as possible until the solvent runs very slowly. 3. In case the material fails to filter properly, it should be transferred to a beaker, warmed slightly until dissolved, precipitated with acetone, purified by the toluene ether method, and subsequently passed through the ether crystallization process. 4. The filtration should be carried out in the cold.

The toluene-ether process consists in dissolving the cadmium chloride salt in a minimum volume of toluene (adding a slight amount of water if necessary). If the toluene fails to dissolve all the material the residue should be centrifuged off. The solution is then treated with 4 volumes of ether containing 1 per cent water. The solution is cooled to 0°C. over night and filtered.

The latter method gives larger yields but removes less of the cephalin and other impurities. It probably removes impurities not taken out by the former method, hence the cadmium chloride compound should be purified by both methods.

Experience shows that in the case of liver lecithin the toluene-ether method should precede the ether crystallization method of purification of the cadmium chloride salt in order to obviate difficulty in filtering from the ether.

One purification by each method should be sufficient to give almost white dry material with an amino content of less than 3 per cent of the total nitrogen present. Such a product may be converted into free lecithin.

Conversion of the Cadmium Chloride Compound into Free Lecithin.

The cadmium chloride salt is dissolved in chloroform and is converted into free lecithin by means of a solution of ammonia in methyl alcohol. 100 gm. of the cadmium chloride salt are dissolved in 300 cc. of warm chloroform and poured into 400 cc. of methyl alcohol containing 20 gm. of ammonia gas. This is added slowly with rapid stirring. The product of reaction is allowed to stand a short time before filtering. The precipitate may be filtered off through a folded filter paper. The chloroform methyl alcohol solution of lecithin is then concentrated under diminished pressure. Near the end of the concentration the material foams considerably for a short time and then the foaming subsides. The vacuum concentration should be carried out at a low temperature. If during the operation a precipitate of fat settles out this should be filtered off. The remaining lecithin is practically free from solvent. It is dissolved in a minimum (5 to 10 cc.) of glacial acetic acid. This is poured into 800 cc. of boiling hot acetone, stirred, and allowed to cool to room temperature. A very small dark precipitate (1 to 2 gm.) settles out. The supernatant liquid is decanted or filtered. The precipitate is slightly soluble in ether and insoluble in acetone but somewhat soluble in ethyl alcohol and more soluble in methyl alcohol.

No. 126.

0.0154 gm. of substance gave on combustion 0.0954 gm. of H_2O, 0.2315 gm. of CO_2, and 0.0114 gm. of ash.

0.1910 gm. of substance used for Kjeldahl nitrogen determination required 3.90 cc. of 0.1 N acid corresponding to 0.00546 gm. of N.

0.2865 gm. of substance gave 0.0390 gm. of $Mg_2P_2O_7$.

$C_{44}H_{87}O_9NP$.	Calculated.	C 65.59, H 10.89, N 1.74, P 3.86.
	Found.	C 61.16, H 10.34, N 2.92, P 3.88.

It contains 10 per cent amino nitrogen.

No. 124.

1.5 gm. of substance were hydrolyzed with HCl, neutralized, concentrated, and made up to 15 cc.

5 cc. of this solution required for Kjeldahl nitrogen determination 5.60 cc. of 0.1 N HCl.

2 cc. of this solution for Van Slyke determination gave 0.57 cc. of N_2 at T = 27°, P = 762.2 mm.

$$\frac{\text{Amino N}}{\text{Total N}} = \frac{10}{100}$$

The liquors are then cooled in a freezing mixture to − 5°C. Frequently at this phase a second small precipitate settles out. A sample of this material analyzed as follows:

No. 122.

0.1024 gm. of substance gave on combustion 0.1098 gm. of H_2O, 0.2216 gm. of CO_2, and 0.0118 gm. of ash.

0.1832 gm. of substance for Kjeldahl nitrogen determination required 3.10 cc. of 0.1 N acid corresponding to 0.00434 gm. of N.

0.2748 gm. of substance gave 0.0464 gm. of $Mg_2P_2O_7$.

$C_{44}H_{87}O_9NP$.	Calculated.	C 65.59, H 10.89, N 1.74, P 3.86.
	Found.	C 59.50, H 12.10, N 2.37, P 4.75.

The mother liquors are concentrated under diminished pressure until all the ether and most of the acetic acid are removed. Water is added a little at a time and the material is shaken or stirred until a thick emulsion of a light brown color is formed. This is poured into 800 to 1,000 cc. of acetone, chilled down to −5°C. It is carefully stirred and allowed to stand at 0 to −5°C. over night, when it is transferred to a crystallizing dish and washed free from excess water by stirring with cold dry acetone. The acetone is decanted off and the lecithin dried in a vacuum desiccator.

From 40 to 45 gm. of pure material may be obtained from 100 gm. of cadmium chloride salt. (Theoretical yield, 81 to 82 gm.) Several samples have been analyzed. They differed little one from another in their elementary composition. The analysis of one of them is as follows:

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of H_2O, 0.2768 gm. of CO_2, and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of $Mg_2P_2O_7$.

$C_{44}H_{87}O_9NP$.* Calculated. C 65.59, H 10.89, N 1.74, P 3.86.
Found. C 64.83, H 11.16, N 1.87, P 4.03.

* This formula represents material consisting of equal parts of two lecithins, each one containing two of the four fatty acids.

II. The Fatty Acids of Lecithin.

For the preparation of fatty acids from lecithin, the material was hydrolyzed 8 to 15 hours with 10 parts of 10 per cent HCl. The fatty acids on cooling appeared as a semisolid cake. They were dissolved in methyl alcohol and precipitated in the presence of ammonium hydroxide with a nearly equal weight of lead acetate dissolved in a minimum quantity of water. After freezing, the mother liquors were filtered off. The lead salts which contained both the saturated and unsaturated fatty acids were extracted repeatedly with boiling ether until further extraction produced only slight precipitate with hydrochloric acid.

The ether solution then contained the lead salts of the unsaturated acids while the ether-insoluble material consisted of the lead salts of the saturated acids. Both fractions were decomposed with HCl, dissolved in ether, washed with water, dried, and the solvent evaporated off.

A lot of 528 gm. of the lecithin cadmium chloride free from amino nitrogen was hydrolyzed with 10 per cent solution of hydrochloric acid. The yield of fatty acids was 223 gm.

Unsaturated Fatty Acids.

These were obtained by extracting the lead salt first by means of acetone and then by means of ether. Each extract was worked over separately. The acetone extract was concentrated and the residue thus obtained extracted with ether. From both of these fractions the acids were liberated and reconverted into the lead salts. These were

again purified and again converted into the free acids. A sample of the acids gave the following iodine and hydrogen values:

0.2907 gm. of substance absorbed 0.435 gm. of iodine by the Wijs method.
0.5141 gm. of substance reduced by Paal's method absorbed 67 cc. of H_2 in 3 hours at 17°C., 759 mm. pressure, or 1.103 gm. of H_2 per 100 gm. of substance.

$C_{18}H_{34}O_2$.	Calculated.	Iodine value	91,	Hydrogen number	0.721.
$C_{18}H_{32}O_2$.	Calculated.	" "	182,	" "	1.447.
	Found.	" "	154,	" "	1.103.

It was later found that this material consisted of two fatty acids, hence it is possible that one was a singly unsaturated, and that the other contained two or more double bonds.

The free fatty acids were finally reduced by Paal's method. The samples of reduced acids obtained from each fraction analyzed as follows:

No. 84 (material obtained from the acetone extract of the lead salts).
0.1020 gm. of substance gave on combustion 0.1198 gm. of H_2O and 0.2830 gm. of CO_2.
No. 85 (material obtained from the ether extract of the lead salts).
0.1012 gm. of substance gave on combustion 0.1190 gm. of H_2O and 0.2828 gm. of CO_2.

No. 84. C 75.96, H 13.19.
" 85. C 76.21, H 13.15.

Since the two fractions proved practically of identical elementary composition they were combined and converted into the methyl esters. These were freed from adhering sulfuric acid by washing with water and finally by recrystallization from methyl alcohol. They were then fractionated by distilling at a pressure of 1 to 2 mm.

The following fractions were obtained.

A.....182–185°C.
B.....175–185°C.
C.....182–195°C.
D.....185–203°C.

Fractions A and D were redistilled and the following fractions were obtained.

From A	A_1	158–165°C.
	A_2	170–182°C.
From D	D_1	182–192°C.
	D_2	187–197°C.

For identification, the esters were saponified with an alcoholic solution of sodium hydroxide. The acids were liberated and converted into the lead salts. The acids were again liberated from the lead salts and analyzed. Fraction A_1 corresponded apparently to pure stearic acid.

Analysis 101.

0.1024 gm. of substance gave on combustion 0.1186 gm. of H_2O and 0.2860 gm. of CO_2.

0.8950 gm. of substance in a molecular weight determination required 6.50 cc. of 0.5 N NaOH.

$C_{18}H_{36}O_2$.	Calculated.	C 75.93, H 12.76.
	Found.	C 76.16, H 12.96.

Molecular weight was 275, that of stearic acid is 284.

The substance melted at 70.5–71°.C, stearic acid melts at 70–71°C.

When this was mixed with some very pure stearic acid melting at 74°C., the mixture melted at 74°C.

Fraction D_2 apparently corresponded to pure arachidic acid.

Analysis 100.

0.1000 gm. of substance gave on combustion 0.1166 gm. of H_2O and 0.2822 gm. of CO_2.

0.9760 gm. of substance neutralized 6.75 cc. of 9.5 N NaOH.

$C_{20}H_{40}O_2$.	Calculated.	C 76.95, H 12.91.
	Found.	C 76.97, H 13.24.

Molecular weight was 314, that of arachidic acid is 313.

The substance melted at 75.5–76°C., arachidic acid melts at 75–77°C.

When this was mixed with some pure arachidic acid melting at 75°C., the mixture melted at 75°C.

Saturated Fatty Acids.

The lead salts which were insoluble in acetone and ether were converted into free acids. These were twice esterified with methyl alcohol. The mixture of methyl esters thus obtained was distilled at a pressure of 1 to 2 mm. into the following fractions:

a.....160–163°C.
b.....159–167°C.
c.....158–172°C.
d.....170–180°C.

and residue.

Fractions a and d were redistilled as follows.

From a	a_1	156–162°C.
	a_2	Residue.
From d	d_1	180–183°C.
	d_2	182–188°C.

Fraction a_1 apparently corresponded to pure palmitic acid.

Analysis 92.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H_2O and 0.2802 gm. of CO_2.

0.8168 gm. of substance neutralized 6.14 gm. of 0.5 N NaOH.

$C_{16}H_{32}O_2$.	Calculated.	C 74.92, H 12.58.
	Found.	C 75.09, H 12.98.

Molecular weight was found to be 266, palmitic acid had a molecular weight of 256.

The melting point was 62°C., palmitic acid melts at 63–64°C.

When this was mixed with some pure palmitic acid melting at 64°C., the mixture melted at 63°C.

Fraction d_2 apparently corresponded to pure stearic acid.

Analysis 94.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H_2O and 0.2802 gm. of CO_2.

0.6686 gm. of substance neutralized 4.82 cc. of 0.5 N NaOH.

$C_{18}H_{36}O_2$.	Calculated.	C 75.93, H 12.76.
	Found.	C 75.72, H 13.53.

Molecular weight was 278, stearic acid had a molecular weight of 284.

The substance melted at 71°C., stearic acid melts at 70–71°C.

When this was mixed with a sample of very pure stearic acid melting at 74°C. the mixture melted at 74°C.

III. Bromine Addition Products of the Unsaturated Acids.

An attempt was made to separate and to characterize the individual unsaturated acids by preparing the bromine addition products.

40 gm. of pure lecithin, which had been prepared from the cadmium chloride salt as described above, were used.

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of H_2O, 0.2768 gm. of CO_2, and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of $Mg_2P_2O_7$.

$C_{44}H_{87}O_9NP$.	Calculated.	C 65.59, H 10.89, N 1.74, P 3.86.
	Found.	C 64.83, H 11.16, N 1.87, P 4.03.

This was hydrolyzed with a 10 per cent solution of hydrochloric acid, the acids were dissolved in ether, washed with water, dried, and the ether evaporated off. The iodine number of the mixed acids was 91.

0.2457 gm. of substance absorbed 0.232 gm. of iodine by the Wijs method.

Average molecular weight of 280.	Calculated.	Iodine value 91.
	Found.	" " 91.

The acids were converted into the lead salts, the unsaturated acids extracted with ether and converted into the free acids. These were dissolved in 18–30° petrolic ether and brominated at 0°C. with 3 cc. of bromine dissolved in petrolic ether.

On freezing to −10° a precipitate was obtained. The mother liquor was concentrated and again cooled to −10°. The combined precipitate was recrystallized from petrolic ether and then recrystallized from ethyl ether.

This gives three fractions: A, the petrolic ether-soluble fraction: B, the fraction insoluble in petrolic ether but soluble in ethyl ether; and C, the fraction insoluble in both solvents. This last fraction contains the material having most bromine (namely, the hexabromides and octobromides, if present). The first fraction should be largely dibromides while the tetrabromides should predominate in the fraction insoluble in petrolic ether but soluble in ethyl ether.

Fraction C (the material insoluble in both solvents) was recrystallized from ethyl ether; the yield was 1 gm. In an open tube melting point determination it darkened, turning black at 200°C. It contracted at 240°C. and decomposed at 243°C. In a closed tube it contracted at 239°C. and melted without decomposition at 243°C. This analyzed as follows:

No. 129.
0.2012 gm. of substance gave 0.2936 gm. of AgBr.

This would indicate a hexabromide.

$C_{20}H_{34}O_2Br_6$.	Calculated for hexabromarachidic acid.	1.0.
$C_{18}H_{30}O_2Br_6$.	Calculated for hexabromstearic acid.	63.2.
	Found.	62.11.

The material was recrystallized from ether. On heating in an open tube it darkened at 180–200°C., contracted at 240°C., and decomposed at 244°C.

It analyzed as follows:

Analysis 130.
0.1068 gm. of substance gave 0.1670 gm. of AgBr.

This corresponds more closely to an octobromide.

$C_{20}H_{32}O_2Br_8$.	Calculated for octobromarachidic acid.	67.80.
	Found.	66.55.

There was not sufficient material for further treatment.

IV. Hydrolecithin from Liver Lecithin.

For the preparation of hydrolecithin 10 gm. of pure liver lecithin free from amino nitrogen were used (Analysis 119 given above). This was reduced by Paal's method. The hydrolecithin produced was recrystallized twice from acetone and once from methyl ethyl ketone.

This analyzed as follows:

No. 128.
0.1074 gm. of substance gave on combustion 0.1082 gm. of H_2O, 0.2554 gm. of CO_2, and 0.0098 gm. of ash.
0.1926 gm. of substance for Kjeldahl nitrogen determination required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.
0.2839 gm. of substance gave 0.0400 gm. of $Mg_2P_2O_7$.

$C_{44}H_{31}O_9NP$.	Calculated.	C 65.30, H 11.33, N 1.73, P 3.84.
	Found.	C 65.03, H 11.29, N 1.74, P 3.86.

A molecular weight determination was made as follows:

1.036 gm. of substance raised the boiling point of 16 gm. methyl alcohol 0.071°C.
0.964 gm. of substance raised the boiling point of 16 gm. methyl alcohol 0.077°C.

$C_{44}H_{91}O_9NP$.	Calculated.	Molecular weight	809.
	Found.	First determination	810.
		Second "	700.

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ON THE NUMERICAL VALUES OF THE OPTICAL ROTATIONS IN THE SUGAR ACIDS.

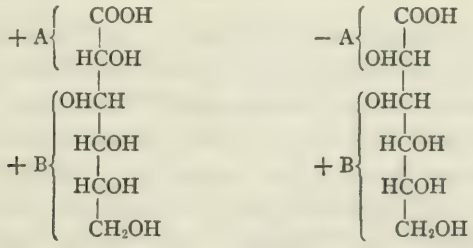
By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 7, 1921.)

In recent years the van't Hoff theory of optical superposition has been on several occasions the subject of discussion. Some writers, Rosanoff, and independently of him Patterson and Taylor have challenged the theory, whereas in a series of very important publications Hudson[1] has demonstrated the validity of the theory and made it the foundation of many important contributions to the chemistry of carbohydrates.

The present writer also has made use of the theory for the purpose of establishing relationships between the configuration of the carbon atom 2 and the optical rotation of epimeric sugar acids. It was found that in a pair of epimeric sugar acids as in gluconic and mannonic acids the molecular structure may be regarded as consisting of two parts, one (A) consisting of carbon atoms 1 and 2, and the other (B) of carbon atoms 3, 4, 5, and 6 as seen from the following.



It is evident that such pairs of epimers may be represented as $\begin{matrix} +A \\ | \\ +B \end{matrix}$ and $\begin{matrix} -A \\ | \\ +B. \end{matrix}$ It was found that in sugar acids and in

[1] Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66; 1917, xxxix, 462; 1918, xl, 813.

2-hexosaminic acids the superposition theory holds to the following extent; the direction of the rotation of the carbon atom 2 is in agreement with the theory in all of the four pairs of hexonic and all of the four pairs of hexosaminic acids. The numerical value of the rotation of carbon atom 2 should according to the theory of van't Hoff be identical for all hexonic and for all hexosaminic acids. This expectation was not realized. In three of the epimers of each group the values are identical, but in the fourth it is markedly different as seen from the following table.

	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{20}$	Phenylhydrazides of	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{20}$
Epichitosaminic	+12.5	+24.37 (10^2)	Gluconic	+14.25	+42.18 (10^2)
Chitosaminic	−12.5	−24.37 (10^2)	Mannonic	−14.25	−42.18 (10^2)
Dextro-xylohexosaminic	+12.5	+24.37 (10^2)	Gulonic	+14.25	+42.18 (10^2)
Levo-xylohexosaminic	−12.5	−24.37 (10^2)	Idonic	−14.25	−42.18 (10^2)
Epichondrosaminic	+12.5	+24.37 (10^2)	Galactonic	+ 8.25	+24.42 (10^2)
Chondrosaminic	−12.5	−24.37 (10^2)	Talonic	− 8.25	−24.42 (10^2)
Dextro-ribohexosaminic	+19.12	+37.28 (10^2)	Allonic	+20.8	+61.56 (10^2)
Levo-ribohexosaminic	−19.12	−37.28 (10^2)	Altronic	−20.8	−61.56 (10^2)

Hudson was stimulated by our findings on the hexonic and hexosaminic acids and developed a more general conclusion, that the direction of the rotation of the carbon atom 2 determines the direction of the optical rotation of the acid. This is seen by mere comparison of our tables without resorting to calculation. Hudson arrived at his conclusion in a very ingenious way. He assumed that the superposition theory holds literally in every detail and he concluded that the magnitude and the direction of the rotation of any one of the four asymmetric carbon atoms in hexonic acid can be calculated from four equations each representing the algebraic sum of the four asymmetric carbon atoms of one acid and of the value of its optical rotation in the following way.

1. *d*-Gluconic acid $+\alpha-\beta+\gamma+\delta=(+12.0)\ (286)=+34.3\ (10^2)$
2. *d*-Gulonic " $+\alpha-\beta-\gamma+\delta=(+13.7)\ (286)=+39.2\ (10^2)$
3. *d*-Idonic " $-\alpha+\beta-\gamma+\delta=(-12.4)\ (286)=-35.5\ (10^2)$
4. *d*-Galactonic " $+\alpha-\beta-\gamma+\delta=(+11.0)\ (286)=+31.5\ (10^2)$

Solving these four equations, the following values are obtained:
$\alpha=+37.3\ (10^2);\ \beta=+3.9\ (10^2);\ \gamma=+1.4\ (10^2);\ \delta=-0.6\ (10^2)$.

Weerman[2] later, working on the amides of sugar acids, corroborated the conclusions of Levene[3, 4, 5] and of Hudson. Hudson alone, and later with Komatsu,[6] repeated the observations on a series of amides. They found the following values.

α-carbon $=+47.25\ (10^2)$; β-carbon $=-14.65\ (10^2)$; γ-carbon $=+0.95\ (10^2)$; δ-carbon $=-2.05\ (10^2)$.

The authors then remark: "It will be noticed that the numerical values decrease as the carbon atom is further removed from the amide end. . . The alternation in the sign of the rotation of the successive carbon atoms is also noteworthy suggesting the alternation in positive and negative affinity that is often ascribed to the carbons in a chain." Inasmuch as we have found that the value of the rotation of carbon atom 2 (α, according to Hudson's nomenclature) is not a constant for all acids, it was suggested that also the magnitudes of rotation of the other carbon atoms are influenced by the configuration of the adjacent carbon atoms. If that were so then the system adopted by Hudson for his calculations may be incorrect and may lead to erroneous conclusions.

We therefore calculated the magnitudes of rotation of each of the four carbon atoms in hexonic and 2-aminohexonic acids from several combinations of four equations. If the magnitudes of rotation of each carbon atom were constant the same values should be obtained from all combinations.

It will be seen from the following table that the results obtained by Hudson and his coworkers are due to special conditions and that some combinations of four equations lead to values of rotation of the

[2] Weerman, Dissertation, Amsterdam, 1916.
[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 623.
[4] Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 367.
[5] Levene, P. A., and Clark, E. P., *J. Biol. Chem.*, 1921, xlvi, 19.
[6] Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xli, 1141.

α-carbon atom (Hudson's nomenclature) which are lower than some one other carbon atom.

In Combinations V, VI, and VII the values for the rotation of every carbon atom remain constant. These combinations are composed of members in which the α-carbon atom is constant and equal to +12.5. On the other hand, in all combinations in which at least one member had the α-carbon atom of the value 19.5 the values for the other carbon atoms were variable. It seemed therefore that the values for β-, γ-, and δ-carbon atoms may possess constant and equal values when derived from the values of Part B of each acid, and not from that of the entire acid.

Calculations again showed that the values for β-, γ-, and δ-carbon atoms remained equal and constant when derived from values of Part B of acids having the rotation of α-carbon atom = 12.5, and not otherwise.

This observation naturally leads to the conclusion that the superposition theory holds only within certain limits.

In certain substances, as in hexonic acids, the vicinity of a certain group (carboxyl) accentuates the rotation of the α-carbon atom to such an extent that the direction of its rotation determines the direction of the rotation of the entire molecule. The correctness of this conclusion with certainty can be demonstrated only on a comparison of the rotation of pairs of epimers.

It is, however, interesting to note that for the series of gluconic and mannonic, galactonic and talonic, gulonic and idonic, the superposition theory holds completely.

Hexosaminic acid.

I.

$[\alpha]_D$	$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +11.625$	$+22.65\ (10^2)$
$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\beta = +\ 2.125$	$+\ 4.14\ (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = +\ 0.125$	$+\ 2.44\ (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = -\ 1.375$	$-26.82\ (10^2)$

II.

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = +\ 6.75$	$+13.15\ (10^2)$
$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\beta = -\ 5.75$	$-11.22\ (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = -\ 4.75$	$-\ 9.23\ (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = -\ 9.25$	$-18.04\ (10^2)$

III.

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = +16.875$	$+32.95\ (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = -\ 1.375$	$-\ 2.68\ (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = -\ 3.375$	$-\ 7.38\ (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = -\ 4.875$	$-\ 9.50\ (10^2)$

IV.

$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +19.5$	$+38.02\ (10^2)$
$\delta + \gamma + \beta - \alpha = -26.5$	$\beta = +\ 7.0$	$+13.65\ (10^2)$
$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\gamma = -\ 4.75$	$-\ 9.23\ (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = -\ 9.25$	$-18.05\ (10^2)$

V.

$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\alpha = +12.5$	$+24.38\ (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = +\ 3.0$	$+\ 5.85\ (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = +\ 1.0$	$+19.50\ (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = -\ 0.5$	$-\ 0.98\ (10^2)$

VI.

$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\alpha = +12.5$	$+24.38\ (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = +\ 3.0$	$+\ 5.85\ (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = +\ 1.0$	$+19.50\ (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = -\ 0.5$	$-\ 0.98\ (10^2)$

VII.

$\delta + \gamma - \beta + \alpha = +10.0$	$\alpha = +12.5$	$+24.38\ (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\beta = +\ 3.0$	$+\ 5.85\ (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\gamma = +\ 1.0$	$+19.50\ (10^2)$
$\delta - \gamma - \beta + \alpha = +\ 8.0$	$\delta = -\ 0.5$	$-\ 0.98\ (10^2)$

Phenylhydrazides of hexonic acids.

VIII.

$[\alpha]_D$	$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = +\ 9.775$	$+27.95\ (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = +\ 8.375$	$+23.93\ (10^2)$
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = +10.625$	$+30.38\ (10^2)$
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = -\ 2.975$	$-\ 8.50\ (10^2)$

IX.

$\delta + \gamma + \beta - \alpha = -15.8$	$\alpha = +10.95$	$+31.32\ (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = -\ 2.65$	$-\ 7.58\ (10^2)$
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = -\ 0.40$	$-\ 1.15\ (10^2)$
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = -\ 1.80$	$+\ 5.16\ (10^2)$

X.

$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = +20.8$	$+59.48\ (10^2)$
$\delta + \gamma + \beta - \alpha = -15.8$	$\beta = +\ 7.15$	$+20.44\ (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\gamma = -\ 0.35$	$-\ 1.00\ (10^2)$
$\delta - \gamma + \beta - \alpha = -15.1$	$\delta = -\ 1.80$	$-\ 5.14\ (10^2)$

XI.

Values of Part B.

	$[\alpha]_D$	$[M]_D$		$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$+24.37\ (10^2)$				
$\delta + \gamma + \beta - \alpha = -26.5$	$-51.67\ (10^2)$	$\delta + \gamma + \beta = -6.75$		$-13.16\ (10^2)$	

XII.

$\delta - \gamma - \beta + \alpha = +\ 8.0$	$+15.6\ (10^2)$	$\delta - \gamma - \beta = -4.5$	$-8.58\ (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$-33.15\ (10^2)$		

XIII.

$\delta - \gamma + \beta + \alpha = +14.0$	$+27.3\ (10^2)$	$\delta - \gamma + \beta = +1.5$	$+2.93\ (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$-21.45\ (10^2)$		

XIV.

$\delta + \gamma - \beta + \alpha = +10.0$	$+19.5\ (10^2)$	$\delta + \gamma - \beta = -2.5$	$-4.88\ (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$-29.25\ (10^2)$		

Solving for XII, XIII, and XIV, the values obtained are $\beta = +3.0$, $\gamma = +1.0$, and $\delta = -0.5$.

Solving for XI, XII, and XIII, the values obtained are $\beta = +3.0$, $\gamma = -4.125$, and $\delta = -5.625$.

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PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

By P. A. LEVENE and G. M. MEYER.

With the Assistance of I. Weber.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 7, 1921.)

The purpose of the preparation of the substance to be described in this communication was developed in a previous publication by Levene and Yamagawa. The interest, as was explained, is both of a theoretical and a practical nature. The question to be answered is the following. Does the stability of the inorganic radicle in a phosphoric ester of a sugar (free or substituted) depend on the position of the inorganic radicle?

The answer to this question is given in the following table of the constants of hydrolysis of three 1-2-acetone phosphoric acid glucoses.

	K
1-2-Monoacetone phosphoric acid glucose	44 (10^{-3})
1-2-Monoacetone-6-phosphoric acid glucose	58 (10^{-3})
1-2-Monoacetone-3- or 5-phosphoric acid glucose[1]	24 (10^{-3})

Substance 1 was prepared from 1-2-acetone glucose, hence the position of the phosphoric acid in it may be in any position from 3 to 6 (inclusive). Substance 2 was prepared in the course of preparation of the derivative from 1-2-3-5-diacetone, hence it is in the main 1-2-diacetone-6-phosphoric acid glucose. The third substance was prepared from 1-2-3-5-diacetone-6-benzoyl glucose. This was converted into 1-2-acetone-6-benzoyl glucose, this further into 1-2-acetone-3- or 5-phosphoric acid-6-benzoyl glucose, and finally the latter into 1-2-acetone-3- or 5-phosphoric acid glucose. Thus in Substance 2 the phosphoric acid radicle is attached to carbon atom 6, and in Substance 3 in position 3 or 5. The differences in the rate of hydrolysis

[1] The second sentence of the last paragraph on p. 324, *J. Biol. Chem.*, xliii, should read: Whereas the fourth was obtained by the action of phosphorus oxychloride on monoacetone glucose, the fifth is formed as a by-product by the action of phosphorus oxychloride on diacetone glucose.

are far beyond the limits of error of the method. It is interesting to note that the rate of hydrolysis of Substance 3 is practically the same as found in the earlier experiments of Levene and Yamagawa on 1-2-acetone-6-benzoyl phosphoric acid. This similarity is easily understood, since the benzoyl group being in position 6 is very labile, hence, in the course of hydrolysis the benzoyl derivative is soon transformed into the monoacetone phosphoric acid glucose. In the same manner is explained the fact that 1-2-acetone-6-phosphoric acid glucose, and 1-2-3-5-diacetone-6-phosphoric acid hydrolyze with the same velocity.

The analytical data on the barium salts of phosphoric esters, here reported, call for a special note. They are not so satisfactory as is desired. However, it has been the experience of this laboratory that when barium salts of pure phosphoric esters are obtainable only in an amorphous form, the analytical results are often not perfect. Thus it was found practically impossible to obtain a perfect agreement between the found and required elementary composition of the amorphous barium salt prepared from the crystalline adenosin-phosphoric acid.

The observations on the phosphoric esters of the 1-2-3-5-methyl glucose and of the 1-3-5-6-methyl glucose are in harmony with those on the esters of the 1-2-acetone glucose.

Thus the rate of hydrolysis of 1-2-3-5-methyl-6-phosphoric acid glucose proceeded normally, giving an average ratio, $K = 44\,(10^{-3})$, which is not far removed from that of the acetone glucose ester having the inorganic radicle in position 6, where $K = 56\,(10^{-3})$. On the other hand, the rate of hydrolysis of 1–3–5–6–methyl–2–phosphoric acid glucose proceeded abnormally. There was noted a rapid hydrolysis at the beginning which reached a maximum after about 6 hours, and the further progress was very slow. The explanation of this result may be the following. The 1–3–5–6–methyl glucose is apparently very difficult to prepare in pure form. The material employed for coupling with phosphoric acid may have contained as an impurity some trimethyl glucose with either carbon atom 1 or carbon atom 6 free. Thus the material derived from this product may be a mixture of two phosphoric esters, one with an inorganic radicle in position 1 or 6, which is labile, and the other in position 2, which is very stable.

The first is decomposed rapidly, whereas the second proceeds at the low rate indicated in the table.

The estimations of the rate of hydrolysis were carried out by Miss I. Weber.

EXPERIMENTAL.

α-Methyl Glucosidophosphoric Acid.

Portions of 10 gm. of dried methyl glucoside are dissolved in 50 cc. of warm, water-free pyridine. This solution is cooled to −20°C. and to it is added, in small portions, a solution of 7.8 gm. (1 mol) phosphorus oxychloride in 20 cc. of pyridine, also cooled to −20°C.

It is important that the pyridine as well as the methyl glucoside should be absolutely dry. For this purpose the substance is dried over phosphorus pentoxide at 100°C. under a pressure of about 1 mm. The pyridine is boiled with reflux over barium oxide for 5 hours and then distilled. The pyridine and phosphorus oxychloride should be well cooled before mixing, and if the pyridine is dry no pyridine hydrochloride will settle out even after standing for some time.

After the phosphorus oxychloride in pyridine has been added to the solution of the glucoside, the reaction mixture is allowed to remain at −20°C. for several hours, during which time a considerable amount of pyridine hydrochloride separates. The reaction mixture is now diluted with 20 cc. of ice water and allowed to come to room temperature when it is further diluted by pouring it into 200 cc. of cold water. An excess of barium hydrate (100 gm.) is added and the pyridine removed by distillation under diminished pressure. The temperature of the bath should not be above +30°C.

When all the pyridine has been removed the solution is made just acid to Congo red with sulfuric acid. The hydrochloric acid is now removed by the addition of 30 gm. of silver sulfate. After shaking this mixture for half an hour it is filtered and the silver removed from the filtrate by hydrogen sulfide and the latter removed by a current of air.

An excess of barium hydrate is added and carbon dioxide passed into the solution until the reaction is neutral to litmus. The solution is now filtered and concentrated under diminished pressure and low temperature. When the volume has been reduced to one-half of the

original, the solution is filtered and the filtrate concentrated to a small volume which is then poured into a large volume of absolute alcohol. For purification the precipitate is dissolved in a small quantity of water, filtered, and reprecipitated with alcohol. The yield was 9.0 gm.

For analysis the substance is dried under diminished pressure at the temperature of water vapor.

0.1042 gm. of substance gave on combustion 0.0866 gm. of CO_2 and 0.0310 gm. of H_2O.

0.2757 gm. of substance gave 0.0746 gm. of $Mg_2P_2O_7$.

0.0982 " " " " 0.0485 " " $BaSO_4$.

$C_7H_{13}O_6H_2PO_3$. Calculated. C 30.7, H 4.8, P 11.0.
Found (calculated Ba-free). C 31.8, H 4.68, P 10.6.

The optical rotation in water was found:

$$[\alpha]_D^{20} = \frac{+1.32^\circ \times 100}{2 \times 1.615} = +81.8^\circ$$

1-2-3-5-Diacetone Glucose.

Fischer's latest method for the preparation of diacetone from β-glucose[2] in a somewhat modified form was employed.

75 gm. of dried β-glucose which is readily prepared by the method of Behrend, are placed in an ordinary glass-stoppered acid bottle with 1,500 cc. of dried acetone containing 1 per cent hydrochloric acid. The bottle was shaken for 24 hours at 30°C. The contents are now dark-colored and nearly all the β-glucose has dissolved. The solution is filtered and neutralized to litmus with sodium methylate, which is prepared by dissolving 4 gm. of sodium in 400 cc. of dry methyl alcohol. When neutral the acetone solutions assume a much lighter color.

The solution is again filtered and concentrated to dryness under diminished pressure. A solid cake is formed and repeatedly extracted with warm petroleum ether from which on cooling diacetone glucose crystallizes. The diacetone glucose is further purified by the method described by Fischer. 600 gm. of β-glucose under proper conditions yield 350 gm. of diacetone glucose.

[2] Fischer, E., and Rund, C., *Ber. chem. Ges.*, 1916, xlix, 93.

Although some monoacetone is also obtained the yield of this substance is comparatively small.

1-2-3-5-Diacetone-6-Phosphoric Acid Glucoside.

The action of phosphorus oxychloride on diacetone glucose depending upon certain conditions yields either a diacetone glucose phosphoric acid, the barium salt of which is soluble in absolute alcohol, or a monoacetone phosphoric acid, the barium salt of which is insoluble in alcohol.

10 gm. of thoroughly dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to $-35°$C. This low temperature is readily obtained by using a mixture of $CaCl_2 + 6H_2O$ and ice. To the sugar solution are added in small amounts 5.4 gm. phosphorus oxychloride in 20 cc. of dry pyridine, likewise cooled to $-35°$. The mixture is now transferred to a bath of $-10°$ and kept therein for 2 hours. During this time crystals of pyridine hydrochloride have separated. The reaction mixture is again cooled to $-35°$C. and about 20 cc. of moist pyridine, also cooled to $-35°$C., added in such small quantities that the temperature will not rise above $-10°$. This is then followed by the addition of a cold solution of 10 cc. each of pyridine and water and finally a small cake of ice.

The reaction product is now allowed to come to room temperature. An excess of barium hydrate is added, the pyridine removed under diminished pressure, and the hydrochloric acid removed by shaking with silver sulfate, and further treated as previously described.

The barium salt of diacetone phosphoric acid is very soluble in alcohol and is not precipitated by ether or acetone. On allowing the alcoholic solution to evaporate to dryness the barium salt is obtained as a fine white powder which when dry is no longer hygroscopic. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried to constant weight under diminished pressure at the temperature of water vapor.

0.1133 gm. of substance gave on combustion 0.1331 gm. of CO_2 and 0.0482 gm. of H_2O.

0.0923 gm. of substance gave 0.0341 gm. of $BaSO_4$.

0.2770 " " " " 0.0625 " " $Mg_2P_2O_7$.

$C_{12}H_{19}O_6H_2PO_3$. Calculated. C 42.25, H 6.18, P 9.10.
Found (calculated Ba-free). C 41.5, H 6.18, P 8.90.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-11^\circ \times 100}{1 \times 4.472} = -2.48^\circ$$

1-2-Monoacetone Phosphoric Acid Glucoside from Diacetone Glucose.

This substance is obtained by the action of phosphorus oxychloride on diacetone glucose when the temperature of the reaction mixture is allowed to rise above $+10^\circ$ during the process of destroying the unutilized phosphorus oxychloride.

10 gm. of dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to -20°. To this is added 5.4 gm. of phosphorus oxychloride dissolved in 20 cc. of pyridine also cooled to -20°. The mixture is allowed to remain at -20° for several hours and then 20 cc. of ice cold water are added. The temperature of the mixture rises to about $+30^\circ$. After allowing it to stand at room temperature for a while it is further diluted with water. An excess of barium hydrate is added and the pyridine removed by distillation under diminished pressure.

The product is treated with silver sulfate and barium hydrate as previously described. The final residue is soluble in water which on the addition of alcohol forms a gelatinous mass. The barium salt is precipitated from its aqueous solution by pouring it into a large volume of dry acetone. The salt is obtained as a fine white powder. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1032 gm. of substance gave on combustion 0.1016 gm. of CO_2 and 0.0394 gm. of H_2O.
0.2679 gm. of substance gave 0.0653 gm. of $Mg_2P_2O_7$.
0.0893 " " " " 0.0424 " " $BaSO_4$.

$C_9H_{16}O_6H_2PO_3$. Calculated. C 35.75, H 6.00, P 10.28.
Found (calculated Ba-free). C 35.9, H 5.61, P 9.30.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.35^\circ \times 100}{1 \times 5.116} = +6.8^\circ$$

1-2-Monoacetone Phosphoric Acid Glucoside from Monoacetone Glucose.

10 gm. of dry monoacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to $-30°$. To this is added 4.4 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine. The reaction mixture is allowed to stand for some time at $-20°$ and treated as described in the previous preparations. The residue is soluble in 95 per cent alcohol which on pouring into a large excess of dry ether precipitates the barium salt of monoacetone phosphoric acid glucoside. The yield was 9 gm.

The substance is dried for analysis under diminished pressure at the temperature of xylene vapor.

0.1103 gm. of substance gave on combustion 0.1056 gm. of CO_2 and 0.0400 gm. of H_2O.

0.2940 gm. of substance gave 0.0744 gm. of $Mg_2P_2O_7$.

0.0980 " " " " 0.0438 " " $BaSO_4$.

$C_9H_{16}O_6H_2PO_3$. Calculated. C 35.75, H 6.00, P 10.28.
Found (calculated Ba-free). C 35.4, H 5.42, P 9.55.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{+20° \times 100}{1 \times 4.000} = +5.0°$$

1-2-Monoacetone-6-Benzoyl Phosphoric Acid Glucoside.

The benzoyl monoacetone glucose was prepared from benzoyl diacetone glucose according to the method of Fischer.

10 gm. of dried benzoyl monoacetone glucose are dissolved in 50 cc. of dry pyridine, and cooled to $-30°$. To this are added in small amounts 4.7 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine, also cooled to $-30°$. The mixture is allowed to stand at $-30°$ for several hours during which time crystals of pyridine hydrochloride have separated. Moist pyridine, cooled to $-20°$, is now added, followed by a cake of ice. The mixture is allowed to come to room temperature, diluted with water, and treated as previously described. The residue is soluble in absolute alcohol from which the barium salt is precipitated by pouring it into a large volume of dry ether. The yield was 10 gm.

For analysis the substance was dried under diminished pressure at the temperature of chloroform vapor.

0.1097 gm. of substance gave on combustion 0.1316 gm. of CO_2 and 0.0426 gm. of H_2O.

0.2941 gm. of substance gave 0.0642 gm. of $Mg_2P_2O_7$.

0.0980 " " " " 0.0394 " " $BaSO_4$.

$C_{16}H_{20}O_7H_2PO_3$. Calculated. C 47.4, H 4.95, P 7.64.
Found (calculated Ba-free). C 42.9, H 5.3, P 8.06.

The optical rotation was found:

$$[\alpha]_D^{20} = \frac{+0.26^\circ \times 100}{1 \times 2.184} = +11.94^\circ$$

From the analysis of the substance and from the fact that it is non-reducing it was supposed that in the process of preparation a small part of the benzoic acid radicle was removed. This assumption was corroborated by the estimation of the benzoic acid on hydrolysis of the substance.

1.000 gm. of barium salt was hydrolyzed with dilute sulfuric acid. The solution was extracted with ether, the ethereal solution dried with anhydrous sodium sulfate and concentrated. The residue consisted of benzoic acid and weighed 0.1376 gm. or 13.76 per cent. Theory required 22.6 per cent.

1-2-Monoacetone Phosphoric Acid Glucose from 1-2-Monoacetone-6-Benzoyl Phosphoric Acid Glucose.

15 gm. of the barium salt of the phosphorated sugar were dissolved in 150 cc. of water and made just acid to Congo red with sulfuric acid. An additional 10 cc. of 2 N sulfuric acid were added and all made up to 200 cc. This was heated in a water bath at 50°C. for 70 minutes. The solution was cooled, filtered, and extracted with ether. The aqueous solution was made alkaline with barium hydrate and filtered from the barium phosphate. Carbon dioxide was passed until the reaction was neutral to litmus. The solution was again filtered and concentrated under diminished pressure at 30°C. The residue was soluble in 95 per cent alcohol and the barium salt precipitated by pouring it into a large volume of dry ether. The yield was 6 gm.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1091 gm. of substance gave on combustion 0.0979 gm. of CO_2 and 0.0420 gm. of H_2O.
0.2811 gm. of substance gave 0.0658 gm. of $Mg_2P_2O_7$.
0.0937 " " " " 0.0446 " " $BaSO_4$.

$C_9H_{16}O_8H_2PO_3$. Calculated. C 35.75, H 6.00, P 10.28.
Found (calculated Ba-free). C 35.67, H 6.24, P 9.48.

The substance does not reduce Fehling's solution until after hydrolysis with dilute acid.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{0.25^\circ \times 100}{1 \times 0.4000} = +6.25^\circ$$

2-3-5-Trimethyl-Methyl Glucoside.

The trimethyl glucoside was prepared by the method of Haworth.[3] Methyl glucoside was methylated in portions of 20 gm. each. After 300 gm. of glucoside had been methylated the combined product consisting of a mixture of the methylated glucosides was subjected to a fractional distillation. The progress of fractionation was controlled by means of the index of refraction as well as by methoxy determinations.

The product was separated into the following fractions:

Fraction.	n	N_D^{20}	CH_3O	Boiling point P = 0.15 mm.
			per cent	°C.
I	46°56.5′	1.4449	60.95	85
II	46°51.5′	1.4450	61.08	90
III	46°41.5′	1.4446	60.00	95
IV	46°01.5′	1.4502	56.67	105
V	45°11.5′	1.4550	52.30	110

The theory for tetramethyl-methyl glucoside requires 61.75 per cent CH_3O and trimethyl-methyl glucoside calls for 52.54 per cent

[3] Haworth, W. N., *J. Chem. Soc.*, 1915, cvii, 8.

CH_3O. Fractions IV and V were again distilled, the main fraction boiling at 108°C., $P = 0.07$ mm., $n = 44° 43'$, $N_D^{20} = 1.45786$, $D_4^{20} = 1.1477$. Found $M_D = 56.06$.

Theory requires 55.917. The yield of this fraction was 137 gm.

Methoxy determination:

0.1803 gm. of substance gave 0.7145 AgI (factor = 13.2) = 52.30 per cent of CH_3O. Theory requires 52.54 per cent.

2-3-5-Trimethyl-6-Phosphoric Acid Methyl Glucoside.

Portions of 10 gm. of the glucoside are dissolved in 50 cc. of dry pyridine and cooled to −30°. To this mixture is added a solution of 6.5 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to −30°C.

The reaction is rather feeble and only after some time pyridine hydrochloride begins to separate. The reaction mixture is allowed to stand for several hours at −20° and then 20 cc. of cold moist pyridine are added. The product is now allowed to come to room temperature and further diluted with ice cold water.

After making alkaline with 60 gm. of solid barium hydrate, the pyridine is removed by distillation under diminished pressure. The reaction product is then treated with silver sulfate and barium hydrate.

The barium salt of this substance is very soluble in alcohol and acetone. It was purified by dissolving it in a small quantity of acetone and precipitating it in a large volume of dry ether. The salt is obtained as a white hygroscopic powder. For analysis it is dried under diminished pressure at the temperature of xylene vapor.

0.1122 gm. of substance gave on combustion 0.0462 gm. of H_2O and 0.1164 gm. of CO_2.

0.0908 gm. of substance gave 0.0382 gm. of $BaSO_4$.

0.2723 " " " " 0.0705 " " $Mg_2P_2O_7$.

$C_{10}H_{19}O_6H_2PO_3$. Calculated. C 37.97, H 6.68, P 9.80.
Found (calculated Ba-free). C 37.50, H 6.12, P 9.45.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-2.36° \times 100}{1 \times 3.062} = +77.07°$$

3-5-6-Trimethyl-1-2-Acetone Glucose.

3-5-6-Trimethyl-methyl glucoside was prepared by methylating 1-2-acetone glucose and converting the product into the glucoside. The methylation of monoacetone glucose had been attempted by Irvine and Scott[4] using the silver oxide and methyl iodide method. The results by this method are not satisfactory. Attempts to methylate the trimethyl glucose obtained after cleaving off the acetone were also unsuccessful. The following method was finally adopted.

Portions of 25 gm. of monoacetone glucose were methylated with an excess of dimethyl sulfate by the method of Haworth. The temperature of the bath should not exceed 55°; the methylation should proceed rather slowly, and the alkali should be always present in a slight excess. To 25 gm. of monoacetone there were used 150 cc. of 30 per cent sodium hydroxide and 90 cc. of freshly distilled dimethyl sulfate. After all the reagents had been added the stirring was continued for one hour at the same temperature. The solution was then cooled and ammonium hydroxide added to destroy any unutilized dimethyl sulfate. The solution was extracted with chloroform and the chloroform extract, after drying over anhydrous sodium sulfate, concentrated under diminished pressure to a syrup. The product of five such methylations was combined, dissolved in ether, dried with anhydrous sodium sulfate, and subjected to a fractional distillation. The largest fraction amounting to 90 gm. boiled at 88–90° at a pressure of 0.03 mm.

0.1025 gm. of substance gave on combustion 0.2042 gm. of CO_2 and 0.0780 gm. of H_2O.

$C_{12}H_{22}O_6$.	Calculated.	C 54.96, H 8.17.
	Found.	C 54.33, H 8.5.

The optical rotation was:

$$[\alpha]_D^{20} = \frac{-1.15^\circ \times 100}{1 \times 4.034} = -28.5^\circ$$

3-5-6-Trimethyl Glucose.

Hydrolysis of the trimethyl acetone glucose was carried out in 75 per cent alcoholic solution containing 0.5 per cent hydrochloric acid and

[4] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 573.

heating for 1 hour at 100°. The solution was shaken with silver carbonate, extracted with ether, and the ethereal solution dried and concentrated to a syrup. This was distilled and a fraction obtained boiling at 147° and $P = 0.05$ mm.

0.1150 gm. of substance gave on combustion 0.2080 gm. of CO_2 and 0.0872 gm. of H_2O.

$C_9H_{18}O_6$. Calculated. C 48.75, H 8.11.
Found. C 49.32, H 8.43.

The optical rotation in alcohol was:

Initial. $$[\alpha]_D^{20} = \frac{-0.75° \times 100}{1 \times 6.874} = -10.95°$$

Final. $$[\alpha]_D^{20} = \frac{-1° \times 100}{1 \times 6.874} = -14.6°$$

It was attempted to convert this product into its methyl glucoside by all the known methods and by several modifications as regards the concentration of catalyst and time of reaction. In every experiment the unchanged material was recovered.

3-5-6-Trimethyl-Methyl Glucoside.

The conversion of trimethyl monoacetone glucose to the glucoside was obtained finally by the following process:

50 gm. of trimethyl acetone glucose were dissolved in 100 cc. of dry methyl alcohol containing 0.1 per cent hydrochloric acid and heated in sealed tubes for 24 hours at 100°C. The hydrochloric acid was removed by shaking with moist silver carbonate and the solution concentrated under diminished pressure to a syrup. This was taken up in ether and dried with anhydrous sodium sulfate. The ethereal solution was concentrated and the syrup fractionated. The larger part distils at 135°, $P = 0.035$ mm. The yield was 20 gm.

0.1510 gm. of substance gave on combustion 0.2758 gm. of CO_2 and 0.1174 gm. of H_2O.

$C_{10}H_{21}O_6$. Calculated. C 50.85, H 8.47.
Found. C 49.8, H 8.7.

Repeated attempts to obtain this glucoside uncontaminated with traces of free sugar were unsuccessful. The product always produced a slight reduction of Fehling's solution.

3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.

10 gm. of 3–5–6–trimethyl–methyl glucoside were dissolved in dry pyridine, cooled to −20°, and a solution of 5.1 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to −20°, was slowly added. There was a slight rise in temperature and after standing at −10° for some time, crystals of pyridine hydrochloride separated. The barium salt, prepared by the method previously outlined, was found to be soluble in alcohol, ether, and acetone. The alcoholic solution was allowed to evaporate. The barium salt which was obtained as a white powder did not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1070 gm. of substance gave on combustion 0.1258 gm. of CO_2 and 0.0570 gm. of H_2O.

0.2918 gm. of substance gave 0.0717 gm. of $Mg_2P_2O_7$.

0.0973 " " " " 0.0274 " " $BaSO_4$.

$C_{10}H_{19}O_6H_2PO_3$. Calculated. C 37.97, H 6.68, P 9.80.
Found (calculated Ba-free). C 38.6, H 7.15, P 8.25.

The barium salt was in the main an acid barium salt, since analysis showed 16.5 per cent of barium; theory requires for the acid salt 17.85 per cent, and for the neutral salt 30.5 per cent barium.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.71° \times 100}{1 \times 2.692} = +26.38°$$

Rates of Hydrolysis.

1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.

1.658 gm. of the barium salt of this substance were dissolved in a small volume of warm water and made up to 50 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 2.832 cc. of 0.1 N H_2SO_4 and 0.168 cc. of water and sealed. The tubes were heated in an oil bath at 100 cc. for the intervals indicated in the following tables. The method of analysis was as described in the paper of Levene and Yamagawa.[5]

[5] Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xliii, 323.

3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.

3.651 gm. of the barium salt of this substance were dissolved in a little warm water and made up to 25 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 1.227 cc. of 0.1 N H_2SO_4 and 0.773 cc. of water and sealed.

The tubes were heated at 100° for the intervals indicated in the following tables.

1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P of total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0014 0.0012	0.0013	0.0009	0.27	3.02
2	0.0030 0.0034	0.0032	0.0022	0.67	7.42
4	0.0062 0.0064	0.0063	0.0044	1.33	14.61
6	0.0082 0.0083	0.0083	0.0059	1.75	19.25
8	0.0099 0.0100	0.0100	0.0070	2.11	23.19
16	0.0160 0.0162	0.0161	0.0115	3.47	38.21
24	0.0199 0.0198	0.0199	0.0139	4.20	46.16

3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P of total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0178 0.0179	0.0179	0.0125	3.37	41.53
2	0.0210 0.0214	0.0213	0.0151	4.09	50.31
4	0.0210 0.0209	0.0210	0.0146	3.96	48.71
6	0.0232 0.0234	0.0233	0.0162	4.39	54.05
8	0.0207 0.0208	0.0208	0.0145	3.92	48.25
16	0.0212 0.0214	0.0213	0.0148	4.01	49.41
		Sample 2.			
1	0.0303 0.0301	0.0302	0.0084	4.46	70.04
2	0.0303 0.0307	0.0305	0.0085	4.50	70.74
4	0.0320 0.0328	0.0324	0.0090	4.89	76.90
6	0.0348 0.0344	0.0346	0.0096	5.11	80.26

1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.

T	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
60	0.0033	0.1044	0.00023
120	0.0080	0.0997	0.00028
240	0.0157	0.0920	0.00028
360	0.0208	0.0869	0.00026
480	0.0250	0.0827	0.00024
960	0.0402	0.0675	0.00021
1,440	0.0500	0.0577	0.00020
Average			0.00024

$a = 0.1077$

3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.

T	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
60	0.0448	0.0629	0.0038
120	0.0530	0.0547	0.0024
240	0.0525	0.0552	0.0012
360	0.0583	0.0494	0.0009
480	0.0520	0.0557	0.0006
960	0.0533	0.0533	0.0003
1,440	0.0692	0.0385	0.0003

$a = 0.1077$

3-5-6-Trimethyl-2- Phosphoric Acid Methyl Glucoside.

Sample 2.

T	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
60	0.0755	0.0322	0.0087
120	0.0763	0.0314	0.0044
240	0.0829	0.0248	0.0026
360	0.0865	0.0212	0.0017

$a = 0.1077$

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MEASUREMENT OF THE GROWTH OF TISSUES IN VITRO.

By ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Two fragments of the same tissue placed in identical media do not always grow at the same rate. Therefore, an acceleration or a retardation of the rate of growth of a tissue placed in an experimental medium cannot be considered as due necessarily to the action of the medium. It may be a mere accident. In order that the differences in the rate of growth of tissues, placed in media of different composition, may be attributed to an action of the medium, it must be ascertained whether these tissues would grow at the same rate, if placed in identical media. In the course of many experiments, it was attempted to find the conditions under which two parts of the same fragment of tissue cultivated in identical media would increase with the same velocity, and to develop a technique by which the increment could be accurately measured.

I. Technique for Obtaining the Growth of Two Fragments of Tissue at the Same Rate.

It is already known that cultures of tissues, made under certain conditions, generally grow at about the same rate.[1] But in several instances, it was found that the differences in area observed after 48 hours reached 20 or even 50 per cent. The causes of these variations were carefully investigated and the reason for the inequality of the growth could always be discovered. Progressively, a number of minute details of the technique were modified and improved, and a method which renders the growth of two fragments of tissue more nearly equal was developed.

[1] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 534–535.

1. Selection of the Tissue.—Connective tissue cells, the growth of which was the subject of the experiments, were obtained from embryonic tissues and from an old strain of fibroblasts. The growth of embryonic tissues is not regular. Moreover, it is almost impossible to cut such fragments in strictly equal parts. However, the heart of chick embryo was found to grow more regularly than other tissues. After 48 hours, a dense ring of fibroblasts has formed around the original fragment and beyond the ring are scattered fusiform or multipolar cells. Sometimes a more or less irregular layer of endothelial cells spreads into the plasma. If the presence of the peripheral cells is disregarded, and the dense ring of fibroblasts is alone taken into consideration, the area of new growth can be measured with some accuracy. The width of this ring of densely packed cells does not vary markedly, and the uniformity of its thickness can easily be ascertained. The growth consists not only of the migration and the multiplication of the cells in the medium, but also of the increase in mass of the original fragment. The increase in area can easily be measured. But, as the tissue is completely opaque, no appreciation of its thickness can be reached. However, if the fragments selected are identical, not only in surface but also in thickness, they grow at about the same rate. This condition may be obtained by the following technique: A heart taken from a chick embryo about 12 days old is opened and washed in Ringer solution. Afterwards, the walls of the ventricles are placed on a glass plate in a little Ringer solution, and square fragments are cut with a sharp cataract knife. When such a fragment is bisected, both parts are of the same thickness, and if their surface is about equal, they grow at the same rate. Often the limits of the new growth and of the primitive fragment become indistinct, and this prevents accurate appreciation of the width of the growth. Although the growth of embryonic heart is more regular than that of other organs, it is still unfitted for accurate measurement. Whenever possible, a strain of connective tissue adapted to life *in vitro* should be employed.

The tissues are taken from stock cultures of a strain of fibroblasts kept for 9 years in plasma and embryonic juices. The area of the fragment must be about 3 by 5 mm. As the tissue is not entirely opaque to light, it is easy to see whether or not its density is uniform.

Zones of greater density, where the cells are often necrotic, can be detected easily by their complete opacity to transmitted light, and they appear as white spots when they are seen by refracted light. The cultures showing these local thickenings are discarded, unless the area of greater density is symmetrical, and can be divided into two equal parts. It is very much easier to select tissues of uniform density when the old strain is used than when the cultures are made of embryonic heart. Another advantage of the old strain is that the edges of the original fragment generally remain visible for 48 hours, and that the inner and outer limits of the new tissue can be recognized. The area of the original fragment remains constant and the entire growth consists of the ring of new tissue in the medium. A third advantage of the old strain of connective tissue is that only fibroblasts grow from it. There are no ameboid cells or layers of endothelial cells, or scattered cells at the periphery of the thick ring of fibroblasts. The outline of the new growth is sharp and can be traced easily. While our experiments were made with a 9 year old strain of fibroblasts, a younger strain can be used with the same results. By placing a fragment of embryonic heart in plasma and embryonic juice, and by resecting the peripheral growth of fibroblasts when it has become very extensive, and cultivating it again, one can obtain an excellent strain of connective tissue. After ten or twelve passages, fibroblasts are practically in pure culture, grow at a regular rate, and can be used for a quantitative study of the growth of connective tissue.

2. Preparation of the Tissue.—The pieces of tissue used for an experiment always consisted of two or four parts of the same fragment. Fragments coming from different cultures may present differences in activity or in structual conditions which would render the rate of growth unequal. When a old strain of fibroblasts is used, the tissue is extirpated from the coagulum by four neat cuts of a sharp cataract knife, made through the peripheral growth. No part of the coagulum not yet invaded by the cells must be left around the tissue. If the incisions are not very sharp, the coagulated plasma may become folded over the cut edge, or a tractus of fibrin still adherent to the remaining coagulum may produce a dislocation of the fibrin network when the tissue is removed from the culture. When the coagulum

has been severed by the knife, the edge of the wound is slightly pushed aside with the blade, in order to make sure that no tractus of fibrin has been left adherent. After the fragment is completely detached from the surrounding coagulum, it is taken on the edge of the knife and placed in Ringer solution. It is examined again in order to make sure that the edges are not folded and that no fragment of mica is adherent to the tissue. With the cataract knife, it is divided into two parts, as equal as possible. It takes a great deal of practice to learn to divide the fragment in the proper way. If the two parts so obtained are not equal in area and in thickness, or if their edges are dilacerated, they must be discarded.

3. Preparation of the Medium.—The preparation of plasma, Ringer solution, and tissue juices has already been described.[2] It was found that still more care should be given to the possible changes of the hydrogen ion concentration of these different substances, and to the impurities which may come from the chemicals used in the salt solutions. Distilled water is redistilled in glass, and then its pH is determined. The purest chemicals are used for Ringer solution. The solutions are kept in Pyrex glassware, or in ordinary glass coated with paraffin. The pipettes are also tested in order to be sure that the alakalinity of the fluid is not increased by their contact. The H ion concentration of the serum and tissue extract may undergo progressive changes. Therefore, they should be carefully tested. Generally, while the pH of embryonic tissue juice is about 7.2 at the time of the preparation, it drops after a few days to 6.5, and if in contact with the pulp remains at that figure for a long time. The pH of plasma remains constant for several weeks. There are other changes which occur in the course of time in serum, and especially in tissue juices and extract, which make them less efficient in promoting growth and which are independent of the changes in the hydrogen ion concentration. It is, therefore, safer not to keep these substances for more than a few days in cold storage.

4. Preparation of the Cultures.—The fluids composing the medium are placed on the cover-glasses at the same time and thoroughly mixed. Plasma should be placed first on the surface of the glass and spread over it, and then the other substances should be added. This detail

[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 532–533.

insures against subsequent partial or total detachment of the clot. Then the tissue fragments are taken from the Ringer solution and placed in the medium. It is important that both media should be not only of the same composition, but also of the same volume and area. The growth of a fragment of tissue in identical volumes of fluid differs when the fluid is spread at the surface of the cover-glass and when it is left in a thick drop. In order that both media may be spread equally, the cover-glass is put on a piece of paper on which a circle has been traced. After the plasma is dropped on the glass, it is spread with the point of a knife in such a way that it covers the subjacent circle. This technique allows the surface of the medium to be identical in both cultures. As cover-glasses are often of varied alkalinity, mica is substituted for glass, or both cultures are made on the same long cover-glass, in order that the conditions may be identical. Immediately after the fragments of tissue are imbedded in the medium, a hollow slide covered with a little vaseline is inverted over the cover-glasses. Care is taken that the capacity of the hollow slides is identical in both cultures. If one is larger, the evaporation of the medium is greater and its concentration increases. It is well known that a change in the concentration of the medium will modify the extent of the growth. In order to eliminate completely this cause of error, both cultures may be made on the same cover-glass and inverted over an oblong hollow slide. The cultures are then sealed at the same time, and placed in the incubator simultaneously and close together, in order that they may not be subjected to different temperatures. They are allowed to grow for 48 hours.

II. Measurement.

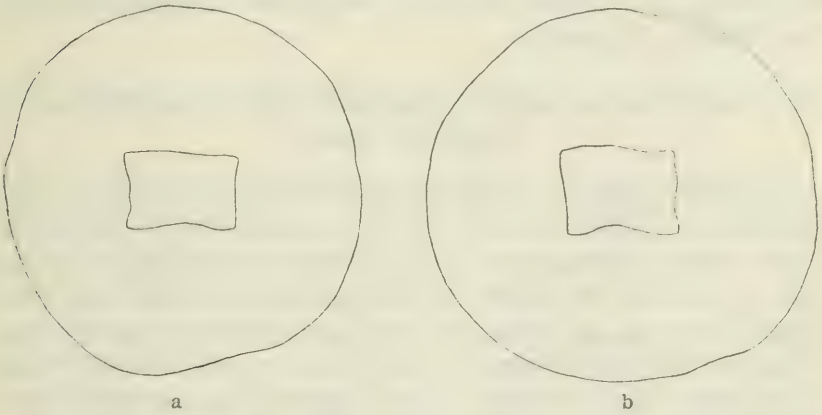
The only means of measuring the growth of a fragment of tissue accurately would be to measure the increase of its weight. Amounts of tissue large enough to be weighed can be easily cultivated *in vitro*, but as the cells cannot be separated from the fibrin of the medium, it is impossible to use this method. The enumeration of the cells contained in the medium would allow an accurate measurement of the increase in number, but connective tissue cells grow in the shape of a tissue, and it is impossible to count them. Therefore, the increase in mass of a fragment of tissue has to be ascertained by indirect meth-

ods. The only practical way was to estimate the increase in mass by the increase in area, the thickness being kept constant. When proper care is taken, the thickness of the new tissue does not vary and can, therefore, be disregarded. The measurement of the areas of new tissue can be made by two different techniques, either by measuring the width of the ring of fibroblasts surrounding the original fragment, or by measuring the area of the new growth. The dense growth of fibroblasts which surrounds a fragment of the old strain of connective tissue after 48 hours is neatly outlined on its outer and inner border. However, when the growth is very active, the inner border, that is the edge of the primitive fragment, becomes indistinct. Generally the side which corresponds in each piece of tissue to the central part of the fragment from which it was derived can be seen distinctly, because it is thicker and darker. It sometimes happens that the growth of the side corresponding to the central part of the mother fragment grows less actively than the three peripheral sides Only the width of the growth of symmetrical sides should be compared in both pieces. The measurements are made with an ocular micrometer. According to the activity of the tissue and the culture medium, the width of the ring of new tissue varies between 0.5 and 8.00. The old strain, when it is in active condition, produces in 48 hours a ring 7 or 8 divisions in width; that is, about 1.4 or 1.6 mm. This measurement does not take into account the increase in size of the original fragment which always occurs when embryonic heart is used. Again, it does not give precise results when, in a very active strain of connective tissue, the limit of new growth and the original fragment after 48 hours have become indistinct. In the case of the old strain, growing at a moderate rate, when the edges of the old plasma remain clear, this method has a sufficient amount of accuracy and the great advantage of being very simple and rapid.

The measurement of the surface of the new growth gives more precise results, but must be made by a more elaborate technique. As soon as the cultures are completed, before the beginning of the growth, the tissues are examined. This shows whether the fragments are still the same size and thickness, whether the edges have been cut neatly, whether no fibrin remains around the tissue, whether there is no folding of the edges of the old plasma, and whether the coagula-

tion of the new medium has taken place in the proper way. If both cultures are not identical in all of these details, they should be rejected. If they are found satisfactory, they are placed in a projecting apparatus and the outline of the fragment is traced on a sheet of paper (Text-fig. 1). Care should be taken to prevent the cultures from being overheated while under the projecting apparatus, and to keep

TEXT-FIG. 1. Experiment 18539; Passage 1657. Areas of two fragments, derived from the same culture, 1 hour after transfer into fresh medium. Area a = 4.5 sq. cm. Area b = 4.1 sq. cm. Reduced about one-third off.



TEXT-FIG. 2. Experiment 18539; Passage 1657. Area of the two fragments shown in Text-fig. 1, 48 hours later. Area a = 56.25 sq. cm. Area b = 49.4 sq. cm. The relative increase of Area a is 11.5 and of Area b 11.2, and the difference is 2 per cent. Reduced about one-third off.

each one outside of the incubator about the same length of time. There is no difficulty either with fresh tissue or with the old strain in making these first drawings. The second drawing is made after 48 hours (Text-fig. 2). The original fragment is again traced and also the outline of the new growth. When the old strain is used, there is generally no difficulty in tracing the outline of the original

fragment, although the cut edges of the old plasma may be covered by so many cells that they have become indistinct. This always happens when heart tissue from a very young embryo is cultivated. The outer edge of the dense ring of fibroblasts can easily be traced. The scattered cells which may be located beyond the limits of the dense growth should be disregarded. If the thickness of the new tissue is irregular, or if the outer edge is irregular and indistinct, the culture should be rejected.

Afterwards, the area of the original fragment and the total area after 48 hours are measured with the planimeter and expressed in square centimeters. The total area minus the area of the original fragment represents the absolute increase of the fragment. This area divided by the area of the primitive fragment is the relative increase of the fragment. The relative increase of the larger fragment divided by the relative increase of the smaller fragment expresses the difference in growth.

III. Results Obtained with Two Fragments of the 9 Year Old Strain of Connective Tissue.

Two series of thirty-two experiments were made, the results of which are shown in Tables I and II. Table I is composed of experiments made with a technique previously described,[3] in which no special care was taken in the selection of the fragments of tissues. In seventeen experiments, the differences between the growth of the two fragments are less than 10 per cent. In nine, it varies between 10 and 20 per cent; in four, between 20 and 30 per cent, and in the last two, the difference in one is 33 per cent, and in the other 51 per cent.

The results of the thirty-two experiments contained in Table II show the value of the improved technique. In twenty-five experiments, the difference in growth is less than 6 per cent; in seven, it varies between 6 and 7 per cent.

These results mean that this technique can be used profitable when the factors acting on the rate of growth are supposed to increase it by more than 10 per cent.

[3] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531–534.

TABLE I.

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
1	16691-1	June 5	1565	2.3	40.7	17.7	1.0	0.0
	16691-2			2.5	44.1	17.64		
2	16694-1	" 5	1565	2.0	30.3	15.5	1.09	0.09
	16694-2			2.2	36.4	16.54		
3	16695-1	" 5	1565	4.7	66.0	14.04	1.12	0.12
	16695-2			5.0	77.3	15.46		
4	17029-1	July 8	1581	3.0	71.9	23.96	1.12	0.12
	17029-2			2.6	55.4	21.3		
5	17030-1	" 8	1581	4.2	91.5	21.78	1.08	0.08
	17030-2			3.4	68.8	20.23		
6	17031-1	" 8	1581	3.1	53.1	17.13	1.10	0.10
	17031-2			3.5	66.5	19.0		
7	17032-1	" 8	1581	3.7	97.0	26.21	1.26	0.26
	17032-2			4.0	80.5	20.12		
8	17059-1	" 12	1583	2.1	34.0	16.2	1.04	0.04
	17059-2			2.0	31.3	15.5		
9	17062-1	" 12	1583	2.1	42.6	20.2	1.04	0.04
	17062-2			2.1	40.9	19.47		
10	17066-1	" 12	1583	3.6	38.6	10.72	1.09	0.09
	17066-2			4.0	39.0	9.75		
11	17075-1	" 12	1582	1.6	39.0	24.47	1.51	0.51
	17075-2			2.0	30.6	15.3		
12	17140-1	" 20	1587	5.8	46.7	8.05	1.07	0.07
	17140-2			5.0	43.3	8.66		
13	17143-1	" 20	1588	3.2	49.1	15.34	1.20	0.20
	17143-2			3.4	43.2	12.7		
14	17145-1	" 20	1588	3.5	36.5	10.45	1.33	0.33
	17145-2			3.7	29.1	7.86		
15	17146-1	" 20	1588	2.5	34.2	13.68	1.15	0.15
	17146-2			2.8	44.3	15.82		
16	17148-1	" 20	1586	3.1	48.3	15.58	1.28	0.28
	17148-2			3.5	42.6	12.17		

TABLE I—*Concluded.*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
17	17200-1	July 26	1591	4.0	49.7	12.42	1.19	0.19
	17200-2			3.5	55.6	14.88		
18	17201-1	" 26	1591	3.5	55.6	14.88	1.06	0.06
	17201-2			3.9	58.7	14.05		
19	17202-1	" 26	1589	3.1	66.3	21.38	1.15	0.15
	17202-2			3.5	64.7	18.48		
20	17203-1	" 26	1589	4.7	63.8	13.57	1.02	0.02
	17203-2			5.2	72.1	13.86		
21	17207-1	" 26	1589	5.5	75.3	13.67	1.19	0.19
	17207-2			6.3	72.7	11.54		
22	17580-1	Sept. 13	1613	6.6	92.2	13.9	1.25	0.25
	17580-2			4.5	78.8	17.4		
23	17586-1	" 13	1613	5.4	67.9	12.5	1.05	0.05
	17586-2			5.4	64.2	11.9		
24	17589-1	" 13	1613	4.7	78.9	16.7	1.13	0.13
	17589-2			5.2	76.7	14.7		
25	17591-1	" 13	1613	4.5	42.1	9.3	1.02	0.02
	17591-2			5.3	50.4	9.5		
26	17592-1	" 13	1613	5.5	81.8	14.8	1.02	0.02
	17592-2			5.8	83.6	14.4		
27	18120-1	Nov. 8	1643	2.5	42.5	17.0	1.08	0.08
	18120-2			2.6	40.8	15.7		
28	18121-1	" 8	1643	4.5	52.0	11.6	1.01	0.01
	18121-2			4.6	51.9	11.3		
29	18122-1	" 8	1643	3.0	43.0	16.3	1.18	0.18
	18122-2			3.2	44.2	13.8		
30	18282-1	" 20	1648	4.9	59.1	12.1	1.03	0.03
	18282-2			5.0	62.6	12.5		
31	18285-1	" 20	1468	6.9	52.1	7.55	1.09	0.09
	18285-2			6.1	49.9	8.3		
32	18289-1	" 20	1648	6.4	64.6	10.1	1.0	0.09
	18289-2			7.0	70.0	10.0		

TABLE II.

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
1	18334-1	Nov. 24	1650	4.2	50.3	12.0	1.06	0.06
	18334-2			3.0	38.3	12.73		
2	18337-1	" 24	1648	4.4	56.2	12.8	1.0	0.0
	18337-2			4.3	55.2	12.9		
3	18340-1	" 24	1650	6.2	47.6	7.7	1.07	0.07
	18340-2			5.4	44.9	8.3		
4	18416-1	" 29	1653	3.5	49.9	14.2	1.05	0.05
	18416-2			3.7	48.6	13.5		
5	18417-1	" 29	1653	4.0	66.9	15.7	1.05	0.05
	18417-2			4.0	68.2	16.0		
6	18419-1	" 29	1653	2.0	30.3	15.5	1.06	0.06
	18419-2			2.2	36.4	16.5		
7	18449-1	Dec. 1	1654	3.0	60.0	20.0	1.04	0.04
	18449-2			3.2	69.9	20.84		
8	18462-1	" 1	1654	2.8	30.24	10.8	1.02	0.02
	18462-2			3.0	33.1	11.0		
9	18463-1	" 1	1654	3.3	50.2	15.5	1.02	0.02
	18463-2			3.9	61.6	15.8		
10	18516-1	" 4	1654	3.9	49.7	12.74	1.02	0.02
	18516-2			2.9	37.7	13.0		
11	18518-1	" 4	1655	3.7	43.3	11.7	1.05	0.05
	18518-2			3.9	44.8	11.43		
12	18522-1	" 4	1656	4.1	53.2	12.97	1.07	0.07
	18522-2			4.1	57.3	13.97		
13	18534-1	" 6	1657	1.8	34.6	19.2	1.02	0.02
	18534-2			1.7	33.3	19.6		
14	18535-1	" 6	1657	3.7	39.3	10.5	1.03	0.03
	18535-2			3.8	42.1	10.01		
15	18539-1	" 6	1657	4.5	51.75	11.5	1.02	0.02
	18539-2			4.1	45.3	11.2		
16	18575-1	" 8	1658	2.3	40.7	17.7	1.0	0.0
	18575-2			2.5	44.1	17.64		

TABLE II—*Concluded.*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
17	18579-1	Dec. 8	1658	5.0	60.1	12.0	1.01	0.01
	18579-2			5.0	61.2	12.2		
18	18580-1	" 8	1658	4.3	50.7	11.66	1.0	0.0
	18580-2			4.2	49.2	11.66		
19	18621-1	" 11	1659	5.2	63.6	12.21	1.02	0.02
	18621-2			5.3	65.5	12.17		
20	18625-1	" 11	1660	3.5	73.0	20.85	1.01	0.01
	18625-2			4.2	77.1	21.0		
21	18628-1	" 11	1660	4.7	64.0	14.01	1.01	0.01
	18628-2			5.0	71.5	14.3		
22	18768-1	" 20	1664	5.1	67.8	13.3	1.06	0.06
	18768-2			5.0	70.1	14.02		
23	18773-1	" 20	1665	4.7	68.3	14.47	1.05	0.05
	18773-2			4.9	67.7	13.69		
24	18777-1	" 20	1667	5.1	77.6	16.1	1.07	0.07
	18777-2			5.0	86.4	17.28		
25	18904-1	" 26	1668	2.5	42.8	17.12	1.05	0.05
	18904-2			2.5	40.8	16.32		
26	18908-1	" 26	1667	4.6	76.8	16.68	1.0	0.0
	18908-2			4.7	73.4	16.68		
27	18909-1	" 26	1667	3.7	68.8	18.52	1.04	0.04
	18909-2			5.0	88.1	17.62		
		1921						
28	19015-1	Jan. 1	1669	4.4	25.0	5.68	1.02	0.02
	19015-2			4.1	23.8	5.8		
29	19024-1	" 1	1670	3.3	62.2	18.88	1.03	0.03
	19024-2			3.2	62.7	19.6		
30	19309-1	" 24	1682	3.5	16.06	3.59	1.03	0.03
	19309-2			4.6	20.5	3.47		
31	19320-1	" 25	1682	3.7	30.7	8.56	1.07	0.07
	19320-2			4.6	36.5	7.96		
32	19495-1	Feb. 7	1683	3.7	32.1	8.67	1.0	0.0
	19495-2			4.4	38.1	8.65		

CONCLUSIONS.

1. A technique has been developed by which two fragments of tissue, put in identical media, can grow at almost the same rate.

2. A method of measuring the growing tissue is described.

3. The differences observed in the rate of growth of two fragments of the 9 year old strain of connective tissue, cultivated in identical media and measured according to this technique, are less than 10 per cent.

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THE MULTIPLICATION OF FIBROBLASTS IN VITRO.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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It is well known that the life of tissues cultivated in the plasma of an adult animal lasts only for a certain period of time. 10 years ago, one of the writers attempted to prevent the death of the tissues living under these conditions.[1] It was supposed that if their waste products were removed by frequent washings, the cells would multiply indefinitely. Every few days, fragments of embryonic heart, cultivated in plasma, were washed in Ringer solution, and placed in a fresh medium. The duration of their life was very much increased, but death still occurred sooner or later. No tissue could be kept alive for more than 3 months.[2] It was evident that the adult plasma did not contain the substances necessary for the indefinite multiplication of fibroblasts. This fact was confirmed by Quagliariello,[3] who injected serum proteins into dogs, and found that they are not used as food by the tissue cells. In the more recent experiments of Kerr, Hurwitz, and Whipple,[4] there was no evidence that serum proteins may be considered as intermediary products between food proteins and parenchyma proteins. Although connective tissue cannot live indefinitely in plasma, it often displays a great activity for several days or weeks. What is the origin of the substances used by the cells in the process of multiplication? The opinion of Lewis,[5] and Ingebrigtsen[6] is that the tissues grow within the limits determined by the amount of food stored up in the body of each individual cell. Burrows[7] also believes that the food material of the tissues is not derived from the medium but from the cells within the fragment. The growth observed in the culture would be a simple transfer of material from the cells of the center of the fragment to those which have migrated into the medium. When growth ceases after a few transplanta-

[1] Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

[2] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[3] Quagliariello, G., *Arch. fisiol.*, 1912, x, 150.

[4] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918–19, xlvii, 356.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

[6] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421.

[7] Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

tions, the sum of the total growth would be less than the original mass. Although no accurate measurements have been made, there is no doubt that a fragment of tissue cultivated in the plasma of an adult animal does not increase materially in size, in spite of the active proliferation of the cells. But the cause of this temporary activity has not as yet been ascertained.

It is also well known that the addition of embryonic tissue juice to the plasma of an adult animal activates the rate of cell division and brings about an immense increase in the mass of the tissue.[8] A strain of fibroblasts derived from a small fragment of embryonic heart has produced about 30,000 cultures in the past 9 years, and is as active today as at the beginning of its life. If this strain had been allowed to grow freely, the volume of tissue so produced would be very much larger than the earth. There is no doubt, therefore, that when embryonic juice is added to adult plasma new cells are made from the substances contained in the medium, and that this process can go on indefinitely.[9]

It then appears that adult plasma alone, and plasma mixed with embryonic juice, differ widely in their action on the growth of tissue. The reason for these differences is still incomp'etely known. It is certain that a mixture of embryonic juice and adult plasma had the power to increase the rate of cell division. But the part played respectively by the constituents of the medium in the multiplication of the cells is not thoroughly understood. The purpose of this article is to investigate whence come the substances used by the fibroblasts cultivated in adult plasma alone, and what constituents of the medium are responsible for the increase of the mass of the tissue when embryonic juices are added to the plasma.

I.

Although the mass of a tissue cultivated in the plasma of an adult animal does not increase, or increases very little, the building of the new cells requires some material which must come from the plasma or from the tissue itself. In order to determine the part played by the plasma, the action of its constituents, fibrin and serum, was separately studied. Connective tissue having been found to grow as extensively in fibrin fixed by formaldehyde as in normal fibrin, the rôle of fibrin must be considered as purely mechanical. In a first series of experi-

[8] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273. Carrel, A., *J. Exp. Med.*, 1913, xviii, 287; 1914, xx, 1.

[9] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

ments, the influence of serum was investigated by measuring the growth of connective tissue in media containing no serum, and serum of different concentrations. In a second series of experiments, the possible action of substances contained in the tissues themselves was examined.

1. Rate of Growth of Tissue in Media Containing Varied Dilutions of Serum.—Fragments of embryonic heart and of a 9 year old strain of fibroblasts were cultivated in media composed of fibrinogen suspension and of Tyrode solution, and containing no serum, or serum in varied dilutions. The serum was taken from the plasma of chickens which had fasted 24 hours. The animals were about 2 years old and in good health. The serum was preserved in paraffined or Pyrex tubes, and its hydrogen ion concentration was measured. Sometimes it was found slightly modified after a few days. The serum was diluted with Tyrode solution sterilized by filtration through a Berkefeld filter. The suspension of fibrinogen was prepared by a technique already known.[10] A medium composed of 10 per cent fibrinogen suspension and 90 per cent Tyrode solution gave a firm coagulum in which embryonic heart tissue could be cultivated without the occurrence of liquefaction. But if a fragment of old strain of fibroblasts was used, liquefaction took place. A little serum had to be added to the medium to prevent this accident. The other media were made of 10 per cent fibrinogen suspension and of mixtures of Tyrode solution and of serum, such that the concentration of serum varied from 2.37 to 90 per cent. The cultures were prepared and measured according to a method previously described.[11] The area of the original fragment was measured immediately after the preparation of the culture, and 48 hours later. The growth was expressed generally by the relative increase of the surface of the tissue; that is, the total area minus the area of the original fragment, divided by the area of the original fragment. The ratio of the relative increase of the experiment to the relative increase of the control permitted the comparison of the growth of cultures belonging to different experiments or groups of experiments. The absolute increase of the tissue was known by the

[10] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.
[11] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

width of the ring of new tissue around the original fragment, measured with a micrometer.

In ten experiments, embryonic heart tissue was cultivated in media containing from 0 to 90 per cent serum (Table I). The growth of every fragment in the experimental medium was compared to the growth of a control in plasma. The increase of the tissues was expressed by the width of the ring of fibroblasts. The experiments in which the layer of new tissue was of unequal thickness were discarded. The figures of Table I show that the tissues grew at least as well in Tyrode solution containing no serum as in plasma. The growth was larger in media, the serum concentration of which varied from 2.37 to 85 per cent, than in plasma. When the amount of growth obtained in a given concentration of serum was compared with that obtained, not in plasma, but in another concentration of serum, it was found that the action of a medium containing 2.37 per cent serum was as marked as that of a medium containing 90 per cent serum. The growth of embryonic heart appeared to be independent of the concentration of the serum as long as the amount of fibrin was not modified. This shows that the cells in their process of multiplication do not make use of the serum to an extent measurable by the present method.

In thirty experiments, an old strain of fibroblasts was used instead of embryonic heart (Table II). It did not grow in a medium composed only of fibrin and Tyrode solution, because the coagulum liquefied in a few hours. There was practically no difference between the amount of growth of the fragments cultivated in media the serum concentrations of which varied from 2.37 to 80 per cent. When fragments of the old strain were cultivated in 10 per cent serum and 50 per cent serum, and kept in the same media for several passages, no difference in the amount of new tissue was observed, even after four passages, as is shown by Text-fig. 1. In several experiments, two fragments of the old strain were studied comparatively in plasma and in 10 per cent serum. The tissues grew at about the same rate, in spite of the great difference in the composition of the medium. The rate of growth decreased rapidly and in about the same way in both media. Death occurred after the sixth or seventh passage. In Text-fig. 2 is shown a typical experiment. The decrease in the rate of growth is more rapid during the first passages in normal plasma

TABLE I.

Growth of Embryonic Heart Tissue in Plasma and Varied Dilutions of Serum, Expressed in Terms of Width of Ring.

Experiment No.	Culture No.	Composition of medium.																				Remarks
		Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	
			0 per cent.		2.37 per cent.		8.3 per cent.		16.5 per cent.		20.5 per cent.		22 per cent.		30 per cent.		33 per cent.		50 per cent.		85 per cent.	
1	18585				1.5		1.8		1.1													
2	18656	0.2	0.4	0.1	0.2	0.2	0.1	0.05	0.2	0.2	1.0					0.2	2.0	0.1	2.0			
3	18700	0.5	Liquefied.	0.4	1.6	0.3	2.0	0.2	0.3													
4	18735	0.6	1.0	0.1	1.6	1.0	1.0	0.7	1.6			0.7	1.3	0.6	1.6							
5	18795	0.8	1.0	0.8	1.1	0.7	1.3	0.8	1.2			0.8	1.2	1.0	1.4							
6	18840	0.5	0.5	0.5	2.0	0.4	1.6	0.7	1.8			0.7	1.5	0.6	0.5							
7	18864	1.5	2.0	1.5	4.0	1.6	4.5	1.3	3.3			1.1	3.5	1.1	1.1							
8	18916	0.8	2.0	0.6	2.2	0.5	3.6	0.6	1.2			0.6	1.1	0.8	1.6					0.9	1.2	
9	18951							0.7	0.5													
10	18962	0.9	2.5	1.2	1.2	1.9	1.7															90 per cent serum control instead of plasma.
Average.		0.6	1.0	0.6	1.7	0.8	1.9	0.6	1.2	0.2	1.0	0.8	1.7	0.8	1.2	0.2	2.0	0.1	2.0	0.9	1.2	

The increase of the tissues is expressed by the width of the ring of fibroblasts which invaded the medium over a period of 48 hours. In the first nine experiments, the control was cultivated in adult plasma. In the tenth experiment, the control was cultivated in 90 per cent serum instead of plasma.

TABLE II.

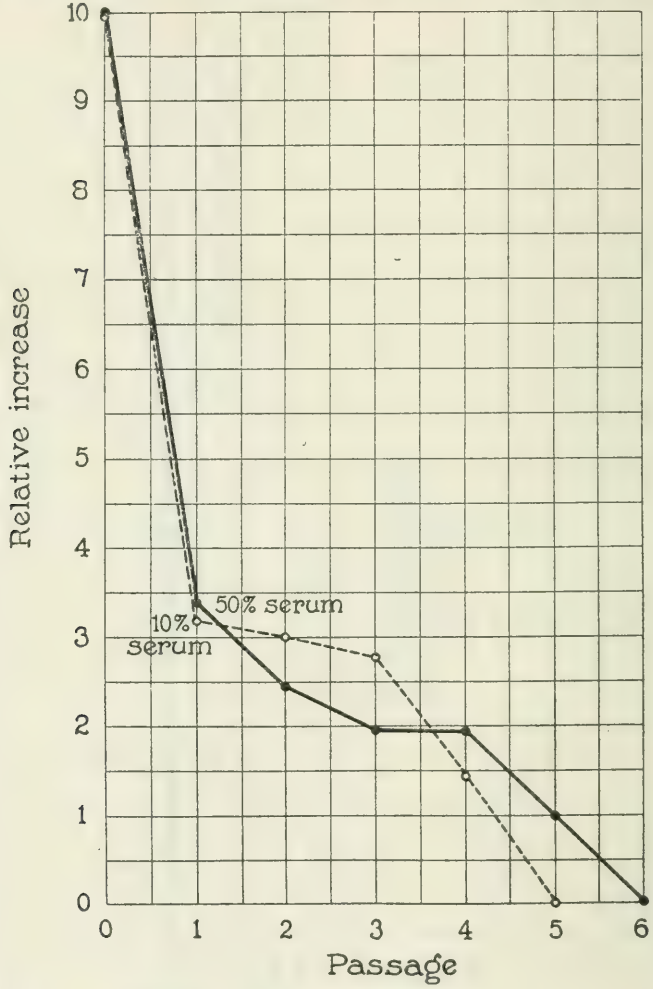
Growth of an Old Strain of Fibroblasts in Plasma and Varied Dilutions of Serum.

Experiment No.	Culture No.	Date.	Composition of medium.													
			Control, plasma.	Experiment, serum. 2.37 per cent.	Control, plasma.	Experiment, serum. 8.3 per cent.	Control, plasma.	Experiment, serum. 16.5 per cent.	Control, serum. 90 per cent.	Experiment, serum. 22 per cent.	Control, serum. 90 per cent.	Experiment, serum. 30 per cent.	Control, serum. 10 per cent.	Experiment, serum. 50 per cent.	Control, serum. 10 per cent.	Experiment, serum. 80 per cent.
		1920														
1	18651	Dec. 13			1.0	1.2										
2	18684	" 20	0.1	0.2	(After 24 hrs.)											
3	18784	" 20	2.5	1.5												
4	18785	" 20	2.0	1.2												
5	18786	" 20			2.2	2.2										
6	18787	" 20			1.6	1.8										
7	18861	" 23	1.7	1.5												
8	18862	" 23	Average,	Average,	1.7	1.5										
9	18863	" 23	2.07	1.6	1.5	1.5										
10	18951	" 28			Average,	Average,	1.1	1.2								
		1921			1.6	1.64										
11	19046	Jan. 3							0.5	0.9						
12	19047	" 3							0.7	1.0						
13	19048	" 3							0.6	0.8						

14	19049	Jan.	3	Control serum.								0.8	0.9				
15	19050	"	3	90 per cent.								0.75	1.0				
16	19051	"	3			Control serum.						0.7	0.8				
17	19067	"	5	Few cells.	1.6												
18	19068	"	5	" "	1.6	91 per cent.											
19	19069	"	5	" "	1.9												
20	19098	"	8		Average,	0.1	1.5										
21	19233	"	17		1.7	0.5	1.1										
22	19234	"	17			0.6	1.0										
23	19235	"	17			0.4	1.3										
24	19237	"	17			0.5	1.3										
25	19364	"	28			Average,	Average,										
26	19365	"	28			0.42	1.22										
27	19366	"	28														
28	19385	"	30											2.0	2.0	1.0	1.0
29	19386	"	30											2.0	2.2	1.1	1.2
30	19387	"	30											2.2	2.2	1.0	1.2
Average.....								1.1	1.2	0.6	0.9	0.75	0.9	2.07	2.13	1.03	1.13

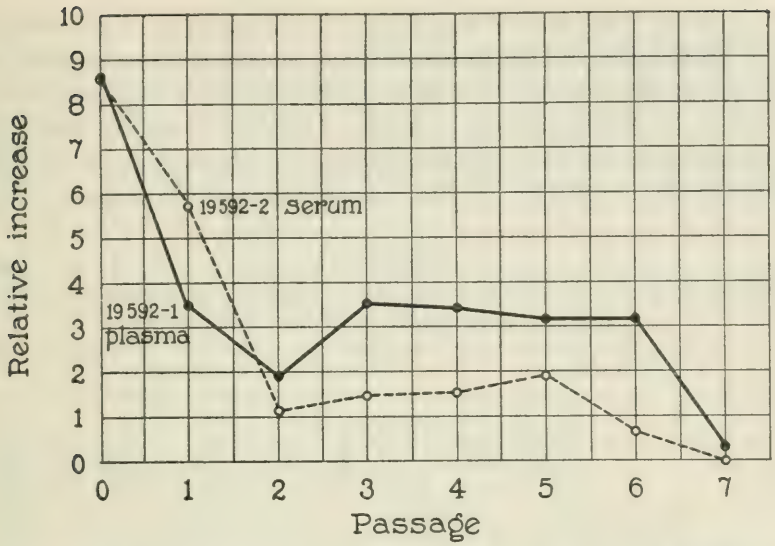
The increase of the tissue is expressed in terms of the width of the ring of new tissue produced in 48 hours.

than in a medium containing only 10 per cent serum. Afterwards, there are only small differences in the amount of tissue produced in both media, and death occurs at the same time. This completes the



TEXT-FIG. 1. Comparison of the rate of growth of two fragments of an old strain of fibroblasts in media containing 10 and 50 per cent serum, in the course of five and six passages (Experiment 19364).

demonstration that the fibroblasts in adult plasma or serum do not make use of the serum proteins.



TEXT-FIG. 2. Comparison of the rate of growth of an old strain of fibroblasts in adult plasma and 10 per cent serum, in the course of seven passages (Experiment 19592).

2. Rate of Growth of Tissue in Plasma.—Fragments of embryonic heart and of an old strain of fibroblasts were cultivated in adult plasma, transferred into new medium every 48 hours, and studied comparatively. The heart was taken from 10 to 14 day old chick embryos, cut in small fragments in a little Ringer solution, and imbedded in plasma. Coagulation always occurred spontaneously after a few minutes, and the growth was measured at the end of 48 hours. During several passages, the tissues displayed great activity, and the surface of the original fragment increased, as well as the width of the ring of new tissue. The rate of growth reached its maximum after a few passages. Then it progressively decreased and death occurred after from ten to thirty passages. The individual differences in the length of life of the cultures were probably due to the nature of the plasma and to the technique used in the transfer of the tissue. The details of two typical experiments are given as an illustration in Table III and Text-fig. 3. The surface of the original fragment, the surface of the new tissue, and the relative increment of the fragment at

each passage were calculated. In the first experiment, the value of the relative increment increased as far as the fourth passage. At the same time, the area of the fragment became larger and reached its maximum at the fifth passage. Later, it progressively decreased and death occurred after the twelfth passage. In the second experiment there was also an increase in the rate of growth of the fragment and

TABLE III.

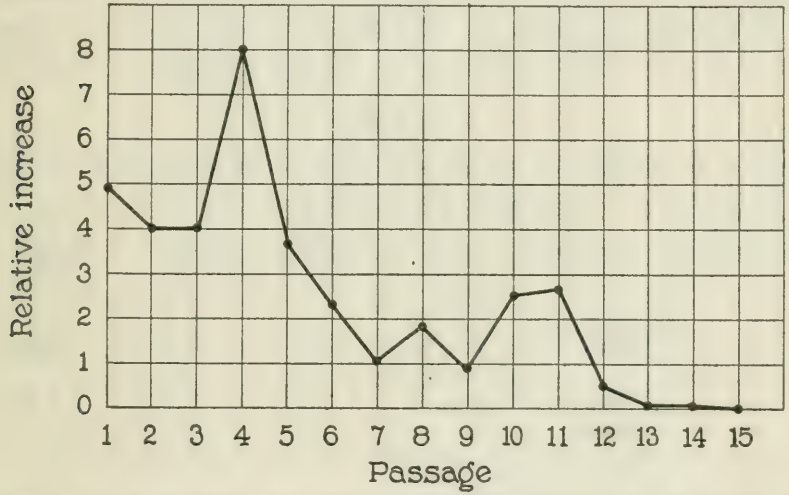
Growth of Embryonic Heart Tissue in Adult Plasma during Fifteen and Eleven Passages (Experiments 18947 and 18948).

Experiment 1.						Experiment 2.					
Passage No.	Culture. No.	Date.	Area of fragment.	Area of growth.	Relative increase.	Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920						*1920*			
1	18947	Dec. 29	1.6	9.1	5.69	1	18948	Dec. 28	1.0	4.9	4.9
2	19006	" 30	1.8	5.7	3.2	2	19007	" 30	1.5	6.0	4.0
		1921						*1921*			
3	19028	Jan. 1	2.1	12.5	5.92	3	19029	Jan. 1	2.5	10.0	4.0
4	19044	" 3	2.5	19.3	7.7	4	19045	" 3	2.1	16.9	8.0
5	19065	" 5	5.1	20.1	4.0	5	19066	" 5	2.6	9.5	3.65
6	19091	" 7	3.5	11.5	3.85	6	19092	" 7	3.3	7.5	2.29
7	19122	" 10	4.4	4.3	1.0	7	19123	" 10	2.8	3.0	1.07
8	19152	" 12	2.7	2.8	1.0	8	19153	" 12	2.3	4.2	1.8
9	19189	" 14	1.2	0.7	0.06	9	19190	" 14	1.9	1.6	0.89
10	19214	" 15	1.5	5.2	3.45	10	19215	" 15	1.3	3.25	2.5
11	19231	" 17	2.1	Few cells.	0	11	19232	" 17	1.7	4.5	2.65
						12	19255	" 19	1.3	0.7	0.5
						13	19280	" 21	0.7	0.4	0.06
						14	19294	" 22	0.5	0.6	0.1
						15	19314	" 24	0.4	0	0

in their size, the maximum of which was reached at the sixth passage. It was impossible to know whether the active multiplication of the cells and the increase in the area of the original fragment during the first passages meant an increase in the volume of the fragment. This point could be ascertained if the tissues were weighed or the cells counted, but the precision of the method which was used is not sufficient to detect a slight and temporary increase of the mass. It

was obvious, however, that the activity of the cultures reached a maximum after a few passages, then decreased until death occurred. It seemed that in the beginning of its life *in vitro* the growth was activated by something which progressively disappeared.

Fragments of a 9 year old strain of fibroblasts were also grown in adult plasma. Previous to the experiment, the strain was kept in a condition of great activity in a mixture of plasma and embryonic juice. Its relative increase in 48 hours was generally from 6 to 10. The fragments were divided and put into plasma. Coagulation of



TEXT-FIG. 3. Growth of embryonic heart in adult plasma during fifteen passages (Experiment 18948).

the medium was obtained by a small piece of fibrin, because fibroblasts living *in vitro* cannot bring about the clotting of the plasma, as embryonic tissues do. Whenever the tissue was cultivated in adult plasma alone, the rate of growth became very much slower. According to the condition of the adult plasma, the decrease in the rate of growth and in the size of the fragment was more or less rapid. In several cases, death occurred after two or three passages, as shown in Table IV and Text-fig. 4. Before the experiment, the relative increase after 48 hours was 10.5. After the first, second, and third passage in adult plasma alone, the relative increase became respec-

tively 1.3, 0.8, and 0. In other experiments, the life of the old strain in adult plasma was longer, and death occurred after six or ten passages (Text-fig. 2). The variations were due to the degree of activity of the strain previous to the experiment, and to the condition of the plasma.

There was a striking difference between the growth of the fragment of embryonic heart and the strain of fibroblasts. As soon as the strain of fibroblasts was placed in plasma alone, the rate of multiplication of its cells decreased (Text-figs. 2 and 4), while under the same conditions the activity of a fragment of embryonic heart increased for several days (Text-fig. 3). The life of the heart was much longer than that of the strain of fibroblasts. These phenomena could not be

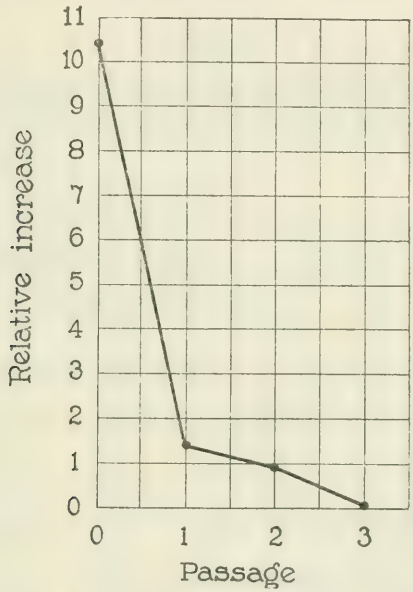
TABLE IV.

Growth of an Old Strain of Fibroblasts in Adult Plasma during Three Passages (Experiment 18907–2).

Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920			
1	18907–2	Dec. 26	5.7	59.3	10.4
	18947	" 28	5.0	6.8	1.36
		1921			
2	19006	Jan. 1	5.3	4.7	0.89
3	19028	" 3	5.3	0	0

attributed to a greater original activity of the embryonic heart, because the rate of proliferation of the fibroblasts of the 9 year old strain is at least as rapid as that of the connective tissue cells of a 10 or 12 day old embryonic heart. They might be due to substances contained in the tissue itself and more abundant in the embryonic tissue than in the strain of fibroblasts. It is known that tissue juices which activate the rate of cell division have also the power of coagulating fluid plasma. Chicken plasma is rapidly transformed into a solid coagulum by a small fragment of embryonic heart, even though the latter has been thoroughly washed in Ringer solution. On the contrary, a fragment of the old strain of fibroblasts does not bring about the coagulation of the plasma. It is, then, possible that embryonic heart contains some of the tissue juice which was found by

one of the writers to be capable of greatly accelerating the rate of cell multiplication,[1] while the strain of fibroblasts lacks it. During the first passages of embryonic heart, the small amount of juice contained in the tissue is probably responsible for the greater activity of the cells. When, after a few days, the original fragment has become surrounded by a dense reticulum of fibroblasts, the rate of growth de-



TEXT-FIG. 4. Growth of an old strain of fibroblasts in adult plasma alone during three passages (Experiment 18907–2).

creases just as it does in a culture of the old strain. It was observed frequently that if a cut were made through the original fragment, after growth had almost ceased, there was a resumption of activity, possibly due to the setting free of some embryonic juice. The activity displayed in adult plasma by both embryonic heart and old strain may be caused by the substances stored within the cells of the tissue, which have the power of increasing the rate of cell division, as is already known.

II.

The indefinite multiplication of fibroblasts, which occurs as soon as embryonic tissue juice is added to adult plasma, may be explained by two different hypotheses: the embryonic juice renders possible the utilization by the cells of substances contained in the serum or the fibrin; or it supplies itself the material required by the cells for their proliferation.

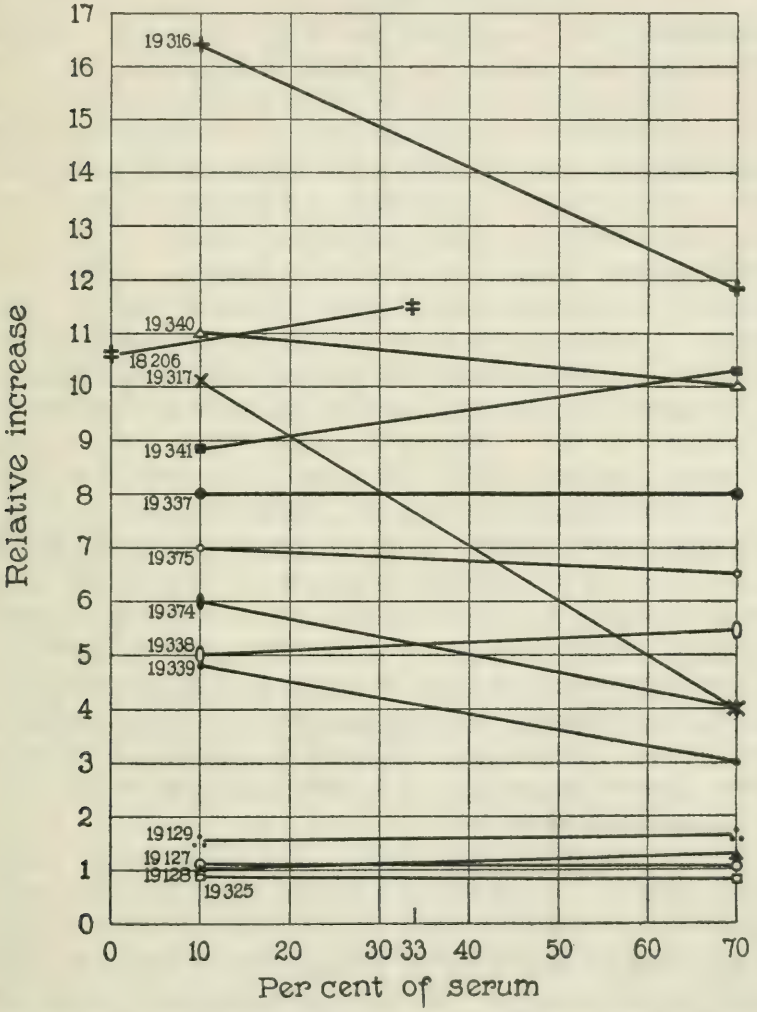
TABLE V.

Growth of an Old Strain of Fibroblasts in a Medium Composed of a Constant Amount of Tissue Juice and 10 and 70 Per Cent Serum.

Experiment No.	Culture No.	Date.	Relative increase.			
			Composition of medium.			
			Control.	Experiment.	Control.	Experiment.
			33 per cent.	0 per cent.	10 per cent.	70 per cent.
		1921				
1	18206		11.5	10.75		
2	19316	Jan. 24			16.4	11.8
3	19317	" 24			10.1	4.0
4	19325	" 25			0.9	0.8
5	19337	" 26			8.0	8.0
6	19338	" 26			5.0	5.4
7	19339	" 26			4.8	3.0
8	19340	" 26			11.0	10.0
9	19341	" 26			8.8	10.3
10	19127	" 10			1.1	1.0
11	19128	" 10			1.0	1.2
12	19129	" 10			1.6	1.6
13	19374	" 27			6.0	4.0
14	19375	" 27			7.0	6.5

Influence of Serum.—In a first group of experiments, it was attempted to find whether serum was used by the cells in the presence of embryonic juice. Tissue juices were obtained by centrifugation of a pulp made of 10 or 12 day old chick embryos. The media were composed of a constant amount of fibrinogen suspension and embryonic tissue juice, and of varied quantities of serum and Tyrode solution. Fragments of the 9 year old strain of fibroblasts were cultivated in these media. The coagula made of fibrin, embryonic juice, and Tyrode

solution liquefied after a few hours in all the experiments but one. In this experiment, the amount of new tissue was practically as large in



TEXT-FIG. 5. Growth of an old strain of fibroblasts in media containing a constant amount of tissue juice and varied dilutions of serum.

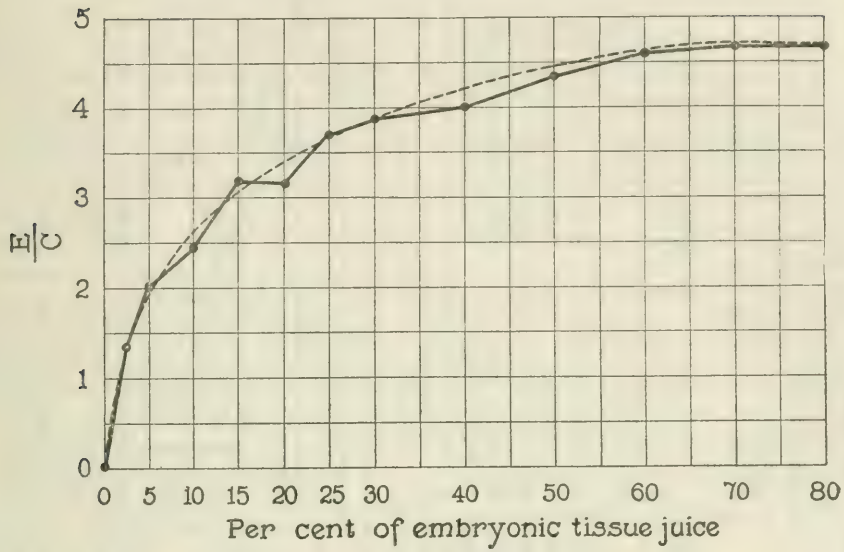
the medium containing no serum as in the medium containing 33 per cent serum. In thirteen experiments the media contained 10

and 70 per cent serum, and their action on the growing tissue was about identical, as is shown by the figures of Table V and by Text-fig. 5. The complete lack of serum, or its presence in low or high concentrations, had no influence on the rate of growth. This fact demonstrated that even in the presence of embryonic juices there was no utilization of the serum by the cells to a measurable extent.

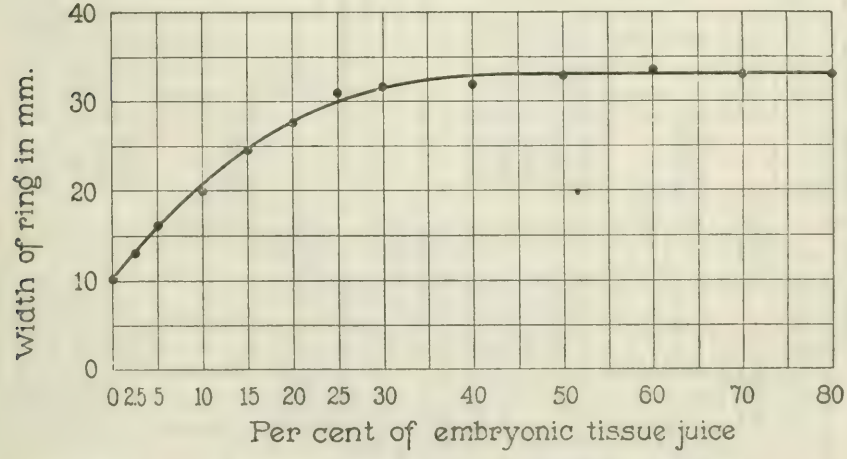
Influence of Fibrin.—In a second group of experiments, the action of the fibrin was studied. Plasma was coagulated by a little tissue extract at the surface of a cover-glass, and the coagulum was fixed in 2 per cent formaldehyde solution in Ringer solution for 1 hour. It became slightly bluish, but remained transparent. Then it was washed for 24 hours in distilled water, and for 24 hours in Ringer solution. It was then placed for half an hour in embryonic juice. The excess of embryonic juice was removed with blotting paper, and fragments of embryonic heart and of an old strain of fibroblasts were cultivated in the clot. The growth assumed the same characteristics and extent as in normal fibrin. In 48 hours, the width of the ring of reticulated fibroblasts was from 5 to 6, which is the same as in normal fibrin and embryonic juice. Since the growth in normal fibrin and in fibrin fixed by formaldehyde is the same, it is practically certain that fibrin plays only a mechanical rôle, and is not used by the cells.

Influence of Embryonic Juice.—In a third group of experiments, the embryonic juice was added to the medium in progressively increasing concentrations, while the amounts of fibrin and serum were kept constant. The tissues responded by considerable differences in the extent of growth, and these differences were the function of the concentration of the juice.

Fifty-five experiments were made. The action of the medium was ascertained by measurement of both the relative and the absolute increase of the tissue (Table VI). The ratios of relative increases of the experiments and of the control tissues were plotted in ordinates, while the concentrations of the juice present in the medium were plotted in abscissæ. The curve (Text-fig. 6) shows that the cells responded to the addition of small amounts of embryonic juice to the medium by a rapid increase of the rate of multiplication. Increases in concentration from 0 to 10 per cent brought about a greater change in the activity of the cells than increases from 10 to 80 per cent. After



TEXT-FIG. 6. Relation between the ratios $\left(\frac{E}{C}\right)$ of the relative increases of the tissue in experiments and controls, and the concentration of embryonic juice in the medium.



TEXT-FIG. 7. Relation between absolute increase of the tissue and the concentration of the embryonic juice in the medium.

TABLE VI.

Relation between the Amount of Growth Expressed in Terms of Relative Increase, Ratios of Relative Increases of Control and Experiments, and of Absolute Increase, and the Concentration of the Embryonic Juice in the Medium.

2.5 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19403	4.5	5.4	1.17	9	13
19404	4.47	6.48	1.44	10	14
19405	4.7	8.13	1.73	9	13
20040	2.66	3.83	1.39	10	12
20041	3.27	4.18	1.27	10	13
20042	4.61	5.27	1.14	9	13
Average.....			1.36	9.5	13

5 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19418	2.85	4.59	1.6	7	15
19419	3.71	9.46	2.58	7	16
20043	2.47	5.93	2.41	10	16
20044	2.21	4.20	1.9	10	18
20045	2.9	5.71	1.97	10	16
Average.....			2.09	8.8	16.2

10 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19437	2.0	5.32	2.66	7	13
19438	2.0	5.61	2.8	7	19
20060	2.87	6.81	2.15	11	22
20061	2.58	5.53	2.14	13	23
20062	2.58	6.21	2.42	12	22
Average.....			2.43	10	19.8

15 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19470	2.5	8.92	3.57	13	18
19471	2.30	9.66	4.2	11	25
20084	3.02	8.55	2.84	11	27
20085	4.78	11.21	2.34	12	28
20086	3.05	10.02	3.28	10	24
Average.....			3.25	11.4	24.4

20 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19484	2.34	5.89	2.53	9	17
19485	3.59	8.52	2.47	12	20
19509	4.2	16.09	3.81	11	33
19510	4.18	15.68	3.80	11	35
19998	3.0	10.97	3.65	12	31
19999	3.17	9.52	3.02	11	27
20000	2.43	7.08	2.9	11	30
Average.....			3.17	11	27.6

25 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19505	2.6	9.6	3.7	10	28
19506	2.83	11.34	4.0	12	27
19507	3.39	13.3	3.9	11	31
20087	2.63	8.93	3.4	12	35
20088	2.56	9.18	3.57	10	33
Average.....			3.7	11	30.8

30 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19511	3.66	15.0	4.07	10	31
19512	3.94	15.0	3.8	10	32
20001	2.33	9.08	3.9	10	33
20002	3.5	11.79	4.17	9	32
20003	2.9	10.0	3.45	14	30
Average.....			3.88	10.6	31.6

40 per cent tissue juice.					
Experiment No.	Relative increase.		Ratio, $\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.
19528	2.3	9.2	4.0	10	30
19529	2.64	10.3	3.9	9	30
19530	2.23	9.6	4.3	10	30
19883	2.74	11.67	4.26	9	35
19884	3.54	12.71	3.6	10	34
Average.....			4.01	9.6	31.8

50 per cent tissue juice.						60 per cent tissue juice.						70 per cent tissue juice.						80 per cent tissue juice.					
	Relative increase.			Width of ring.			Relative increase.			Width of ring.			Relative increase.			Width of ring.			Relative increase.			Width of ring.	
Experiment No.	Control.	Experiment.	$\frac{E}{C}$ Ratio.	Control.	Experiment.	Experiment No.	Control.	Experiment.	$\frac{E}{C}$ Ratio.	Control.	Experiment.	Experiment No.	Control.	Experiment.	$\frac{E}{C}$ Ratio.	Control.	Experiment.	Experiment No.	Control.	Experiment.	$\frac{E}{C}$ Ratio.	Control.	Experiment.
19531	2.36	9.20	3.9	10	26	19544	2.52	11.6	4.6	12	33	19546	2.47	11.36	4.6	9	33	19620	2.33	10.62	4.58	13	33
19533	2.74	13.2	4.8	10	31	19545	2.85	13.3	4.66	7	34	19547	2.82	13.4	4.75	10	33	19621	1.93	9.08	4.7	12	33
19886	2.17	9.77	4.5	14	36																		
19885	2.49	9.95	4.0	13	34	Average.....			4.6	9.5	33.5	Average.....			4.65	9.5	33	Average.....			4.64	12.5	33
20150	2.76	12.91	4.67	10	35																		
20151	2.10	8.74	4.16	11	34																		
Average.....			4.37	11.2	32.7																		

the concentration of the juice in the medium had reached 40 per cent, no further increase was observed when larger amounts were added. The results were checked by measurement of the width of the ring of the new tissue; that is, by comparing the absolute instead of the relative increases (Table VI). The arithmetic mean of the width of the ring of new tissue of several experiments, made with each dilution of juice, was plotted in ordinates, and the concentration of the juice in abscissæ (Text-fig. 7). The curve (Text-fig. 6) showed the same rapid increase of the rate of growth when a small amount of juice was added to the medium. There was no doubt that the substances used by the fibroblasts came from the embryonic juice and that the activity of the fibroblasts was a function of the concentration of the embryonic juice in the medium.

III.

SUMMARY.

The results of the investigation of the cause of the multiplication of fibroblasts *in vitro* may be summarized as follows: Although the life of fibroblasts in the plasma of an adult chicken which has fasted for 24 hours is not permanent, their proliferation is very active for some time. Are the substances used by the cells in their multiplication supplied by the plasma or by the tissue itself? In media composed of a constant amount of fibrin and of a mixture of Tyrode solution and serum in varied concentrations, the amount of growth appeared to be independent of the concentration, and even of the presence of serum. Serum was evidently not used by the cells. It was also found that fibrin is not utilized. This fact explains the results of the experiments of Lewis, who showed long ago that embryonic tissue can grow extensively in Locke solution. Then, the material from which the new cells are built must come from the tissue itself, as was previously supposed by Lewis, Ingebrigtsen, and Burrows. A comparative study of the growth in adult plasma of embryonic heart and of a 9 year old strain of fibroblasts led to the hypothesis that traces of embryonic juice stored in the original fragments are responsible for the activity manifested by the tissues during their temporary life.

When embryonic juice is added to the plasma of an adult chicken, the rate of multiplication of the fibroblasts increases and their life *in vitro* becomes permanent. Does the presence of embryonic juice determine the use by the cells of substances contained in adult plasma? The tissues were cultivated in media containing a constant amount of fibrin and embryonic juice, and varied concentrations of serum. The rate of growth was found to be independent of the amount of serum contained in the medium. It was also observed that the rate of growth in fibrin fixed in formaldehyde solution did not differ from that in normal fibrin. This fact demonstrated that embryonic juice does not give to the cells the power of using the constituents of plasma. When fragments of the 9 year old strain of fibroblasts were cultivated in media containing a constant amount of serum and fibrin, and varied concentrations of embryo juice, the rate of growth was found to be a function of the concentration of the embryonic juice in the medium. It was, therefore, evident that the material employed by the fibroblasts in their indefinite multiplication *in vitro* was supplied by the embryonic juice.

IV.

CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments and within the limits of accuracy of the method, the temporary multiplication of fibroblasts cultivated in the plasma of an adult animal is not due to the serum. It may be attributed to the presence of a small amount of embryonic juice within the tissue itself.

2. The indefinite multiplication of fibroblasts in a medium composed of adult plasma and of embryonic juice is due neither to the serum nor to the fibrin. It depends entirely on substances contained in the embryonic juice.

3. There is a definite relation between the rate of growth and the concentration of the embryonic juice in the medium.

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CICATRIZATION OF WOUNDS.

XI. Latent Period.

By ALEXIS CARREL, M.D., and P. LECOMTE du NOÜY, Sc. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 26, 1921.)

The latent or quiescent period of cicatrization[1] extends from the time of traumatism to the beginning of contraction. During this stage of apparent inactivity, the mechanism which will bring about the redintegration of the tissues is progressively set in motion. A study of the latent period may lead to a better understanding of the secondary causes which directly or indirectly derive from the injury acting as primary cause, and are instrumental in starting regeneration. The purpose of this article is to examine the duration of the latent period, its transition to the period of contraction, and the characteristics of the curve expressing it.

I.

Method.

The experiments were made with wounds of geometric shape, obtained by excision of a flap of skin in the dorsal region of dogs. The animals were of medium size, short haired, and of quiet temper. 24 hours previous to the operation, they were given a warm bath after the hair had been clipped. Later on the same day, the skin of the dorsal region was washed a second time with soap and water. After the animal had been etherized, the skin was shaved, carefully washed with soap and warm water, and covered for 10 minutes with compresses soaked in 75 per cent alcohol. Then the animal was placed on the operating table and the skin was painted with tincture of 10 per cent iodine, which was allowed to dry for 10 minutes.

[1] Carrel, A., *J. Am. Med. Assn.*, 1910, lv, 2148.

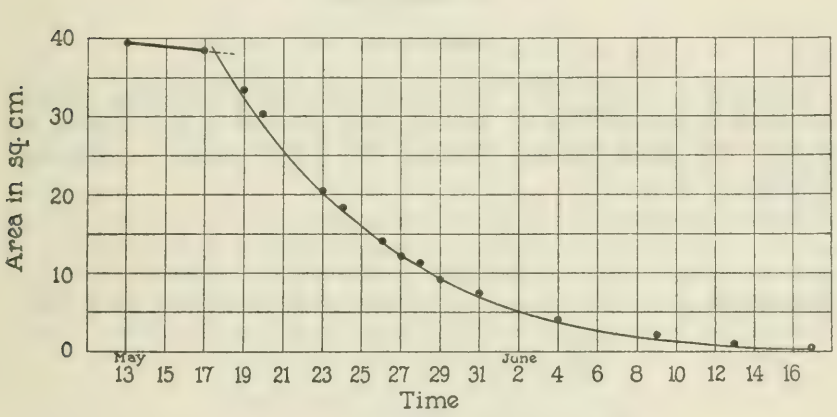
The wounds were generally obtained by resection of a rectangular flap of skin. In some cases, circular flaps were extirpated by means of a sharp edged tube, 2 cc. in diameter. Hemostasis was secured by compression with gauze pads, or by temporary clamping of the small vessels. No ligatures were used, because after a few days they become centers of colonization for the bacteria existing on the surrounding skin. The measurement of the wounds was made in two different ways. In the earlier experiments, the width of a rectangular wound was ascertained with a compass and its variations were studied, instead of the change in the total area. In the more recent experiments, the area of the wound was measured, according to a technique previously described,[2] and expressed in square centimeters. An accurate appreciation of the area required a great deal of care. It is well known that the skin of the dog is not adherent to the aponeurosis, and is very mobile. This causes the size of the wound to become modified by slight changes in the position of the animal. Therefore, in spite of the precautions which were taken, variations occurred in the area of the wounds which were due only to experimental errors. At the end of the latent period, when the edges of the wound had become fixed to the bottom by granulation tissue, the method was much more accurate.

As the purpose of the experiments was to study the latent period under ordinary conditions, the dressing consisted only of talcum powder, paraffin, or plain gauze. The bacterial condition of the surface was ascertained merely by examination of films. Often the wound could be maintained in a condition of surgical asepsis, that is of mild infection, when the dressing was properly fixed to the skin. Infection was usually carried from the surrounding skin to the wound by the gauze, which moves about on the surface of the skin due to the constant movements of the animal. This could be prevented by stitching a gauze pad to the skin itself. Then a thick cotton pad was applied, fixed by bandages, and protected by a shirt.

Seven experiments are briefly described, and the appearance of the latent period is shown by Text-figs. 1 to 7. The width of the wound in centimeters, or its area in square centimeters, is plotted in ordinates and the time in abscissæ. The latent period is designated

[2] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 430.

by the heavy line. The light line expresses the area of the wounds calculated by the formula of du Noüy,[3] which, of course, does not apply to the widths. In Text-figs. 5 to 7 the curves are arbitrary. The observed area, or width, is represented by the heavy points. In the first experiment, the latent period and the two subsequent periods are described, and the curve expresses the complete phenomenon from the excision of the flap to the last stage of epidermization. In the six other experiments, the latent period and the beginning of the contraction period only are shown.



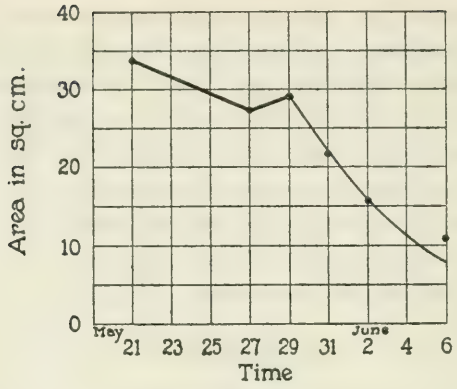
TEXT-FIG. 1.

Experiment 1. Latent Period and Periods of Contraction and Epidermization.— Very old female dog, about 13 years of age, No. 1. May 13, 1919. Resection of a rectangular flap of skin on dorsal region. Dressing with dry gauze. Subsequent dressings, dry gauze after washing with neutral sodium oleate. Dakin's solution compress when bacteria appeared in the films.

Date.....	May 13	17	19	20	23	24	26	27	28	29	31	June 4	9	13	17
Observed area....	39.4	38.7	33.5	30.2	20.3	18.7	14.3	12.2	11.5	9.2	7.7	4.0	2.7	1.1	0.5
Calculated area...					20.0	18.3	14.2	12.2	11.0	9.2	7.1	3.8	1.6	0.8	0.3

For the calculated curve, the index i was equal to 0.058.

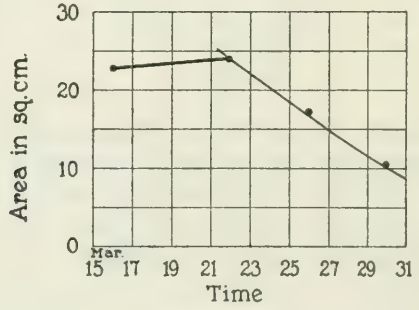
[3] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.



TEXT-FIG. 2.

Experiment 2. Latent Period and Abrupt Beginning of Contraction.—Female dog, about 2 years old, No. 2. May 21, 1919. Excision of a rectangular flap of skin. Dry gauze dressing. Washing with sodium oleate in subsequent dressings.

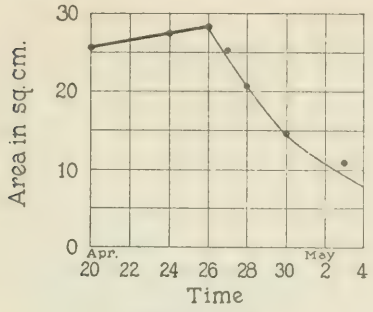
Date	May 21	27	29	31	June 2	6
Observed area	33.7	27.4	29.0	21.7	15.8	11.0



TEXT-FIG. 3.

Experiment 3. Latent Period and Period of Contraction.—Male dog, about 2 years old, No. 3. March 16, 1920. Excision of a rectangular flap of skin. Dry gauze dressings.

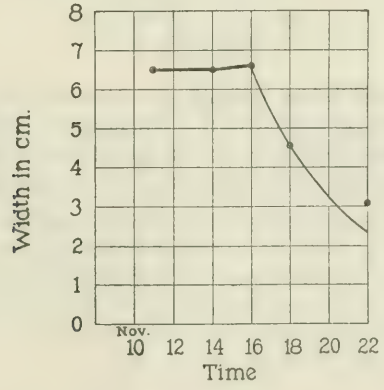
Date	Mar. 16	22	26	30
Observed area	23.0	24.0	17.3	10.6



TEXT-FIG. 4.

Experiment 4. Latent Period and Period of Contraction.—Male dog, about 1 year old, No. 4. April 20, 1920. Excision of flap of skin. Dry gauze dressing. Washing with neutral sodium oleate in subsequent dressings.

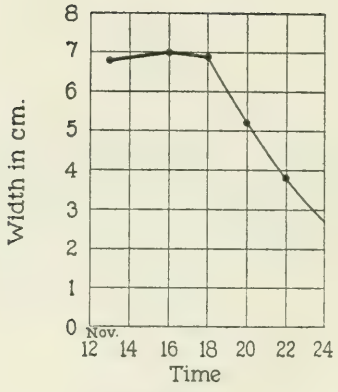
Date	Apr. 20	24	26	27	28	30	May 3
Observed area	25.8	27.4	28.4	25.1	20.7	14.7	11.0



TEXT-FIG. 5.

Experiment 5. Latent Period and Abrupt Beginning of Contraction.—Young male dog, about 11 months old, No. 5. November 11, 1907. Resection of a rectangular flap of skin in dorsal region. Measurement of the width of the wound with a compass. Talcum powder dressing. November 16. Beginning of granulations. Subsequent dressings, dry gauze.

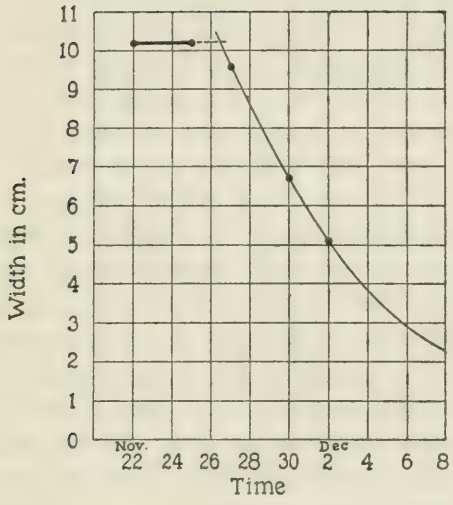
Date	Nov. 11	14	16	18	22
Width of wound	11.0	6.5	6.6	4.6	3.1



TEXT-FIG. 6.

Experiment 6. Latent Period and Abrupt Beginning of Contraction.—Young male dog, about 13 months old, No. 6. November 13, 1907. Resection of a rectangular flap of skin. Measurement of the width of the wound with a compass. Talcum powder dressing. November 18. Small irregular granulations.

Date	Nov. 13	16	18	20	22
Width of wound	6.8	7.0	6.9	5.2	3.8



TEXT-FIG. 7.

Experiment 7. Latent Period and Period of Contraction.—Young male dog, about 10 months old, No. 7. November 22, 1907. Resection of a rectangular flap of skin. Measurement of wound with a compass. Talcum powder dressing. November 27. Granulations began.

Date	Nov. 22	25	27	30	Dec. 2
Width of wound	11.0	10.2	9.6	6.7	5.1

II.

DISCUSSION.

The duration of the latent period varied from 5 to 7 days. It was found, in the course of many other experiments, that the limits of variations may be wider. While the subsequent stages of cicatrization are not affected by external factors, it appears that the latent period may be easily modified in its duration by many local causes, such as mechanical irritation of the tissues, infection, and even by the diet of the animal, as was shown by Clark.[4] The experiments described above deal only with the latent period under ordinary conditions of mild bacterial and mechanical irritation, which was generally obtained by dry gauze dressings. During the first stage of healing, the area was generally found to remain constant. However, in Experiment 7 an increase of the surface, and in Experiment 5 a decrease followed by an increase of the surface, were observed. These changes were probably due to errors of observation. As long as the edges of the wound were not fixed to the bottom by granulating tissue, a slight modification of the tension of the surrounding skin could modify the area considerably. The appearance of the granulation tissue generally announced the end of the latent period. But there was no definite relation between both phenomena. The end of the latent period was marked not only by the growth of granulation tissue, but by an abrupt beginning of the contraction period, which immediately acquired its maximum velocity. In Experiments 4 and 7, there was a transition period which lasted possibly 24 hours, and during which the contraction started slowly. As a rule, the onset of contraction was sudden and a period of maximum activity succeeded the period of complete quiescence.

The formula of du Noüy was found to apply accurately to the beginning of the period of contraction. On the graphs, it may be seen that the light line representing the calculated area follows the observed area almost constantly. There was a perfect coincidence between the calculated and the observed surfaces in Experiment 1, even during the first hours of the contraction. An examination of the seven graphs shows that the phenomenon could not be accurately

[4] Clark, A. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 117.

expressed by a curve presenting a point of inflexion. So far, this fact has not been emphasized, and, on account of an insufficient description of the conditions of the experiments, certain curves, in previous publications, were misleading.[5] Recently, Fauré-Frémiet and Vlès[6] attempted to apply to the cicatrization process the equation expressing the monomolecular autocatalytic reaction used by Robertson[7] in his studies on growth. As a consequence of this hypothesis, the curve of the areas must pass by a point of inflexion, in conformity with the equation

$$\frac{S}{S_o - S} = e^{KSo\,(T - t_{\frac{1}{2}})}$$

which necessitates that the rate should be maximum at the time $t_{\frac{1}{2}}$ where $S = \frac{S_o}{2}$. One of the curves calculated in this manner agrees fairly closely with that of du Noüy. The other presents a point of inflexion; that is, a maximum in the rate of healing during the period of contraction. There is evidently a close relation between this curve and the observed curve of an experiment previously reported.[5] But the point of inflexion of this observed curve was due to a mere accident. The wound was old and infected, and had begun to cicatrize under a plaster of Paris splint. The observed curve showed the variations of the rate of cicatrization both before and after normal cicatrization had really begun. As these details were not mentioned in the paper, Fauré-Frémiet and Vlès[6] could not help being misled by this accidental fact. Although their formula and the formulas published by de Beaujeu[8] and Lumière[9] show a lack of agreement with the observed facts, there is no doubt that the curve representing the normal process of cicatrization may be expressed by more than an equation, as has already been pointed out by du Noüy.[10]

[5] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 432.

[6] Fauré-Frémiet, E., and Vlès, F., *Compt. rend. Acad.*, clxviii, 363.

[7] Robertson, T. B., Principles of biochemistry, Philadelphia, 1920, 475.

[8] de Beaujeu, J., *J. Exp. Med.*, 1917, xxvi, 81.

[9] Lumière, A., *Bull. Acad. méd.*, 1918, lxxix. series 3, 213; *Rev. chir.*, 1917, liii, 656.

[10] du Noüy, P. L., Recherches expérimentales et application des méthodes de mesure et de calcul à un phénomène biologique: la cicatrisation, Thèse de Paris, Paris, 1917.

But these equations must apply as well to the beginning of the contraction period as to the end of the epidermization period. A glance at the figures and at the chart expressing the observed and calculated areas of Experiment 1 shows that the coincidence is almost perfect and quite satisfactory, in view of the percentage error necessarily involved in experimental wounds on dogs.

Several equations have already been applied by du Noüy to the curve of cicatrization. The first[11] was expressed as follows:

$$T = K_1 \operatorname{Log}_e \frac{S_o}{S} + 2K_2 \left(\sqrt{S_o} - \sqrt{S}\right)$$

The second[12] has proven more satisfactory:

$$S = S_o\, e^{-K\left(T + \frac{T^2}{2p}\right)}$$

The figures calculated by this last equation coincide almost exactly with those calculated by means of the well known extrapolation formula:[3]

$$S_n = S_{n-1}\left[1 - i\left(t + \sqrt{t + nt}\right)\right]$$

They applied to the first stage of contraction as accurately as to the later periods of cicatrization.

III.

CONCLUSIONS.

1. The latent period of cicatrization varies generally from 5 to 7 days.

2. It stops abruptly and contraction starts with its maximum velocity.

3. The formula of du Noüy applies to the beginning of the contraction period as well as to the subsequent periods.

[11] du Noüy, P. L., *J. Exp. Med.*, 1917, xxv, 722.

[12] du Noüy, P. L., *J. Exp. Med.*, 1919, xxix, 329, Text-fig. 4

[Reprinted from The Journal of Experimental Medicine, November 1, 1921, Vol. xxxiv, No. 5, pp. 425–434.]

CICATRIZATION OF WOUNDS.

XII. Factors Initiating Regeneration.

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 24, 1921.)

The nature of the factors which initiate cicatrization after an injury is not as yet exactly known. The resumption of cell proliferation in the wounded tissues of an adult animal may be attributed to the removal of resistance to growth, in consequence of the defect resulting from loss of tissue.[1] In other words, the removal of the products of growth, that is of a portion of the tissues, immediately reinaugurates the growth process, just as the removal of the products of a balanced chemical reaction at equilibrium immediately reinitiates the forward reaction.[2] This means that regeneration, being a direct consequence of the injury, is started by forces within the organism. But the same phenomenon may also be logically attributed to the action of an external factor. According to this hypothesis, the cells would be directly stimulated to growth and multiplication by forces without the organism, acting on tissues deprived of their natural protection by the injury.

I.

Effect of Protection against Irritation.

If regeneration is a direct consequence of the loss of tissue and initiated by an internal factor, the cicatrization of a wound protected against all external irritation must take place normally. But if this hypothesis be not true, the wound should not begin to cica-

[1] Welch, W. H., *Science*, 1897, v, 813.

[2] Robertson, T. B., Principles of biochemistry for students of medicine, agriculture and related sciences, Philadelphia, 1920, 482.

trize. It was observed in 1908 that the latent period of cicatrization of a wound dressed with dead connective tissue or plasma clot was abnormally prolonged. This fact suggested that regeneration was not initiated directly by the loss of tissue and that, if the surface of the wound were effectively protected against mechanical, chemical, and bacterial irritations, the setting in motion of the process of cicatrization would be indefinitely postponed. In order to ascertain in what measure the onset of regeneration could be delayed by adequate protection of the surface of the wound, five experiments were performed. Two circular wounds of equal size were made on the dorsal region of dogs, according to a technique previously described.[3,4] The control wound was covered with a paste containing chloramine-T in a concentration which had been shown to be non-irritating for the tissues, and to keep them in a sterile condition.[5] The experimental wound was dressed with subcutaneous connective tissue, excised from the lumbar region of a dog and kept in cold storage. Circular flaps, slightly larger than the wound and about 0.5 cm. thick, were prepared and fixed to the surface of the experimental wound by a few stitches. Both wounds were protected by a pad of dry gauze, sutured to the skin. Then the dressing was completed by a few other gauze pads, a large amount of cotton, a bandage, and a shirt. The animals were examined after a period of time varying from 13 to 25 days. As the examination involved the removal of the stitches holding the inner dressing to the skin, and also of the stitches fixed to the connective tissue placed on the wound, necessitating a considerable disturbance of the wound, the experiment was stopped after the second or third dressing.

The results of the five experiments are summarized in Table I. Experiment 1 was unsuccessful because the gauze pads slipped from the surface of the wounds and infection occurred. 14 days after the operation, no difference was found in the condition of both control and experimental wounds. Experiments 2 and 3 succeeded partly. The protection given to the wounds by the connective

[3] Carrel, Alexis, and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

[4] All operations were performed under ether anesthesia.

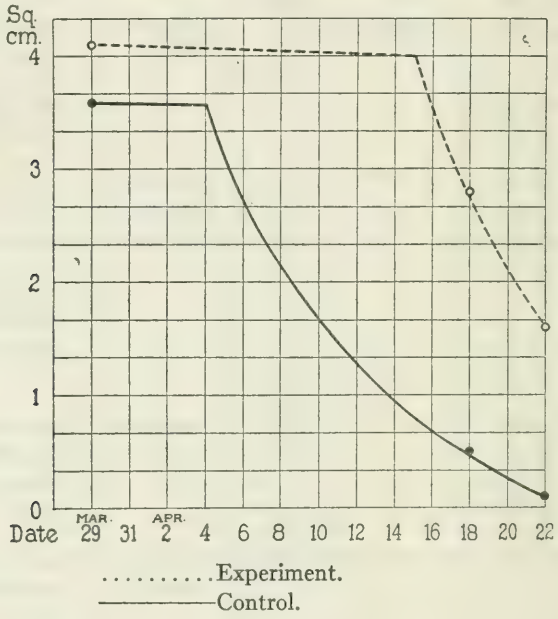
[5] Carrel, Alexis, du Noüy, P. L., and Carrel, Anne, *J. Exp. Med.*, 1917, xxvi, 279.

TABLE I.

Action of Connective Tissue Dressing on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
1	1	Apr. 5	4.0		Chloramine-T.	4.6		Connective tissue.	Connective tissue was heated at 56°C. for 3 hrs.
		" 19	1.9	16	"	1.4	1		Inner dressing slipped from both wounds.
2	2	Mar. 29	3.6		"	4.1		Connective tissue.	Text-fig. 1.
		Apr. 18	0.5	0	"	2.8	0	Chloramine-T.	Inner dressing slipped from both wounds. Connective tissue dressing partially disappeared.
		" 22	0.1	0	"	1.6	0	"	
3	3	Mar. 31	4.0		"	3.9		Connective tissue.	Inner dressing slipped. Infection of both wounds.
		Apr. 19	0.7	∞		2.8	∞		
4	4	Mar. 31	3.5		Chloramine-T.	4.7		Connective tissue.	Text-fig. 2. No displacement of dressing.
		Apr. 7	3.0	0	"				
		" 19	2.0	0	"	4.7	0	Dry gauze.	
		" 25	1.7	0	"	5.1		" "	Appearance of granulation tissue.
5	5	" 7	5.2		"	4.2		Connective tissue.	Text-fig. 3. Connective tissue heated at 56°C.
		" 20	3.3	0	"	4.0	0	Chloramine-T.	No displacement of dressing.
		" 25	3.0	0	"	4.5	0		Appearance of granulation tissue.

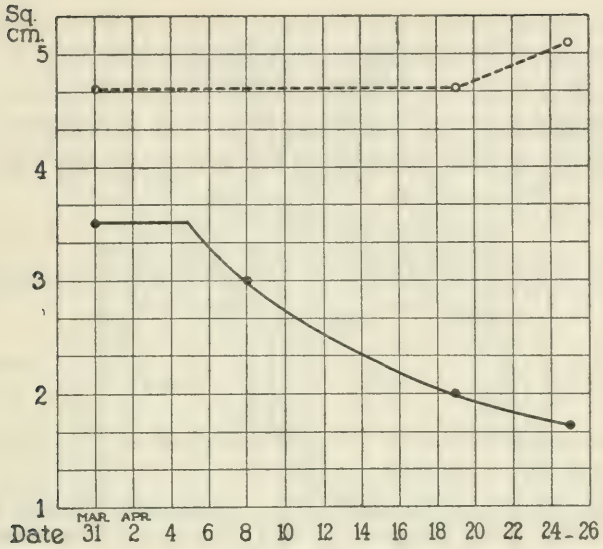
tissue dressing was incomplete. When the wounds were inspected for the first time, 19 and 20 days respectively after the operation, the experimental wound was no longer covered by the connective tissue dressing, and cicatrization had started. In Experiment 2, the curve expressing the progress of regeneration showed that the latent period had lasted very much longer in the wound protected by connective tissue than in the control wound (Text-fig. 1). The duration of the latent period was probably 17 days, while in the control it was 6.



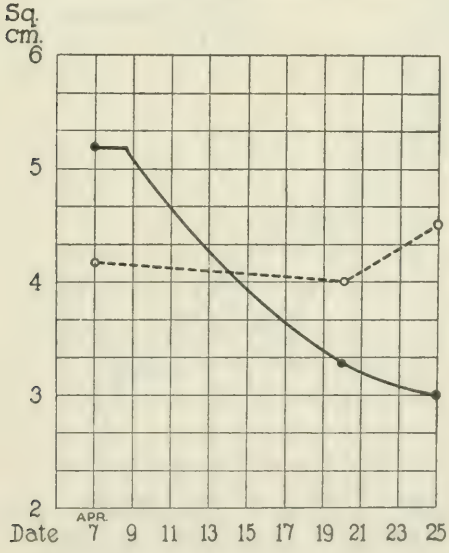
TEXT-FIG. 1. Experiment 2, Table I.

Although the connective tissue dressing did not remain at the surface of the wound for a long time, its effect was, however, manifest.

More significant results were obtained in Experiments 4 and 5. The connective tissue dressing remained exactly where it was applied and the surface of the wound was really protected against all outside irritation. The examination of the wounds was made 25 days after the operation in Experiment 4, and 18 days after the operation in Experiment 5. The period of contraction had not yet started and the area at that time was as large as at the time of the operation (Text-



TEXT-FIG. 2. Experiment 4, Table I.



TEXT-FIG. 3. Experiment 5, Table I.

figs. 2 and 3). It was a striking fact that a wound, effectively protected by a non-irritant dressing, did not show any evidence of cicatrization 25 days after the operation.

II.

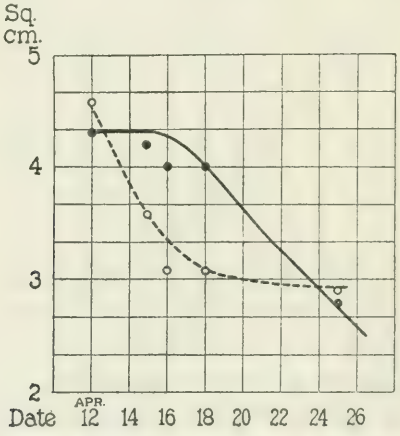
Effect of Irritants.

In a second series of experiments, it was investigated whether the application of mild irritants on the surface of the wound would

TABLE II.

Action of Turpentine on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
6	6	Apr. 12	4.3		Chloramine-T.	4.6		Turpentine.	Text-fig. 4.
		" 15	4.2	0	Dry gauze.	3.6	0	Dry gauze.	No displacement of dressing.
		" 16	4.0	0	" "	3.1	0	" "	
		" 18	4.0	0	" "	3.1	0	Turpentine.	
		" 25	2.8	0		2.9	1		



TEXT-FIG. 4. Experiment 6, Table II.

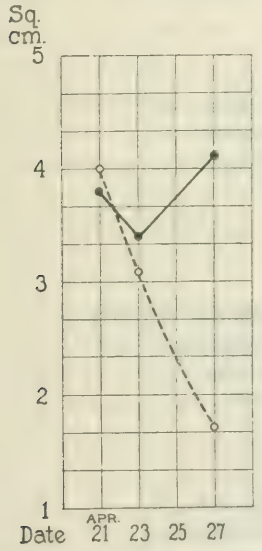
shorten the latent period. The experimental wound was covered by a gauze pad, soaked in turpentine and fixed to the edges of the skin by a few stitches, while the control wound was dressed with chloramine

paste. Both wounds were protected by gauze pads, sutured to the skin. The latent period of the experimental wound was very much shortened, lasting less than 2 days, while the latent period of

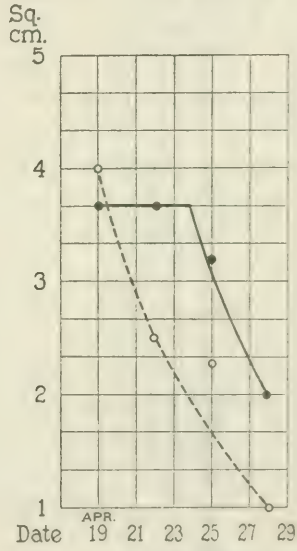
TABLE III.

Action of Chick Embryo Pulp on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
7	7	Apr. 21	3.8		Chloramine-T.	4.0		Chick embryo pulp.	Text-fig. 5.
		" 23	3.4	0	"	3.1	5	Dry gauze.	
		" 27	4.1	0	"	1.7	0	" "	



TEXT-FIG. 5. Experiment 7, Table III.



TEXT-FIG. 6. Experiment 10, Table IV.

the control wound lasted for about 5 or 6 days (Table II and Text-fig. 4).

In Experiment 7, chick embryo pulp was used instead of turpentine (Table III). 6 days after the operation, the contraction of the

control wound had not started. On the contrary, in the experimental wound contraction began after a very short time, less than 2 days

TABLE IV.

Action of Staphylococcic Infection on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
8	8	Apr. 19	5.0		Chloramine-T.	4.0		1:100 dilution of staphylococcus suspension.	Mild infection; no edema; slight discharge.
		" 21		8	"		78	Dry gauze.	
		" 22	4.0	0	"	3.5	45	" "	
		" 25	3.2	0	"	3.3	24	" "	
		" 28	2.8	0	"	1.8	13	" "	
9	9	" 19	4.0		"	3.7		1:10 dilution of staphylococcus suspension.	
		" 21		4	"		8	Dry gauze.	
		" 22	3.7	0	"	3.4	85	" "	
		" 25	3.4	0	"	3.9	1	" "	
		" 28	2.8	0	"	1.7	20	" "	
10	10	" 19	3.7		"	4.0		1:50 dilution of staphylococcus suspension.	Text-fig. 6.
		" 22	3.7	0	"	2.5	50	Dry gauze.	
		" 25	3.2	0	"	2.3	0	" "	
		" 28	2.0	0		1.0	41	" "	
11	11	" 21	4.0		Chloramine-T.	3.5		Pure staphylococcus cultures.	
		" 23	2.6	0	"	2.4	6	Dry gauze.	
		" 25	2.5	0	"	2.2	0	"	
		" 28	2.3	0	"	0			

(Text-fig. 5). 6 days after the operation, the area of the experimental wound was about 50 per cent smaller than that of the control wound.

In four experiments (Table IV), the wounds were infected with staphylococci. Varied dilutions of a 24 hour culture of staphylococci in bouillon were used for inoculation. The control wound was dressed with chloramine paste, while the experimental wound was inoculated with 0.05 cc. of the dilution of staphylococcic culture, and dressed with dry gauze. The wounds remained in a condition of slight infection without swelling of the edges or abundant suppuration. The duration of the latent period was decreased, and often reduced to less than 2 days, as shown in Experiment 10 (Text-fig. 6).

III.

SUMMARY.

As long as the wounds were protected by a connective tissue dressing against mechanical, chemical, and bacterial irritations, no evidence of cicatrization was found. The complete or partial failure of four experiments was due to the slipping of the inner dressing from the wound, mechanical irritation by the gauze, and infection. In the two experiments in which the connective tissue was maintained at the surface of the wound, there was no beginning of cicatrization, although 25 and 18 days respectively had elapsed since the operation, while in the control wound the duration of the latent period did not exceed 5 or 6 days. The experiments were interrupted after the second or third inspection, on account of the technical impossibility of again applying to the wounds a non-irritant dressing. It is probable that the wounds could have been kept for a much longer time in a condition of quiescence. While it is not known whether cicatrization could be prevented for an indefinite period, there is no doubt that the mechanism of regeneration is not set in motion at the usual time, when all external irritations are suppressed. It appears, therefore, that under ordinary conditions, cicatrization is not initiated by an internal factor.

On the contrary, the application of turpentine, chick embryo pulp, and staphylococci decreased markedly the length of the latent period, which was often reduced to less than 2 days. This fact demonstrated the importance of external factors in the initiation of cicatrization. It seems that the mechanism of regeneration has

become adapted to the ordinary conditions of life of the animals. A small wound will begin to cicatrize sooner if slightly infected, as practically always happens, than if it were thoroughly protected by a non-irritant dressing.

IV.

CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments, a wound, protected by a non-irritant dressing, shows no granulation tissue or beginning of contraction for 25 days at least.

2. Local application of certain irritants, such as turpentine, chick embryo pulp, and staphylococci, reduces the duration of the latent period to less than 2 days.

3. Regeneration is apparently initiated, not by an internal, but by an external factor.

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REMOTE RESULTS OF COMPLETE HOMOTRANSPLANTATION OF THE CORNEA.

By ALBERT H. EBELING, M.D., and ANNE CARREL.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 34.

(Received for publication, June 6, 1921.)

Homoplastic transplantation of the cornea in its entire thickness has been attempted many times with practically constant failure. However, if this operation be performed under proper conditions, the transplanted fragments can remain transparent, and the curvatures of the cornea normal. This fact was demonstrated by Zirm, who resected part of a leucoma and substituted for it a flap of normal cornea taken from the enucleated eye of a boy.[1] About a year after the operation, the patient could see through the transplanted cornea. Although it had been implanted in scar tissue, the flap had remained transparent. Such a result is exceptional, and many other attempts have been unsuccessful. As the inadequacy of the technique was probably responsible for the failures of the operation, it would be important to improve the method of transplanting the cornea in such a way that it could be used as a routine procedure in cases of leucoma. The purpose of the experiments described in this paper was to develop a better technique for homoplastic transplantations.

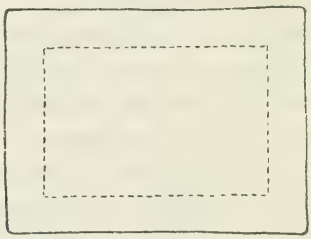
The technique consisted essentially in using a very large rectangular flap, fitted like the door of a safe into the edges of the corneal opening, and in fixing it securely in its position by stitches. This technique had been developed by Carrel in 1912, in the course of unpublished experiments on autotransplantation of the cornea. We attempted to adapt it to homoplastic transplantation, and to observe the results over a long period of time.

1. Preparation of the Eye.—The experiments were performed on cats. Cultures in bouillon were made of the secretion of the con-

[1] Zirm, E., *Arch. Ophth.*, 1906, lxiv, 580; *Wien. klin. Woch.*, 1907, xx, 61.

junctiva and only animals whose conjunctiva was found to be sterile were used. The hair covering the palpebra and the skin surrounding the eye were cut with scissors. Several days previous to the operation, the eye and the surrounding skin were washed with mercury bichloride at 1:5,000. Immediately before the operation, the surrounding skin and that of the palpebra were washed with alcohol, and afterwards tincture of iodine was applied.

2. Preparation of the Graft.—The graft was taken from the eye of a cat under ether anesthesia. The outline of a rectangular flap 6 by 8 mm. was traced on the surface of the cornea with a sharp cataract knife. The surface was incised to a depth corresponding to about half the cornea. Then the internal edge of the flap was dissected a distance of 1 mm. The anterior chamber was opened, the incision of the posterior part of the cornea continued with the scissors, the flap removed, and put in olive oil until it was used. Its anterior surface was 6 by 8 mm., while its posterior surface was only 4 by 6 mm. (Text-fig. 1).

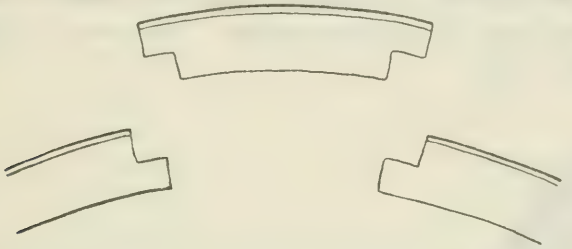


TEXT-FIG. 1. Anterior aspect of flap.

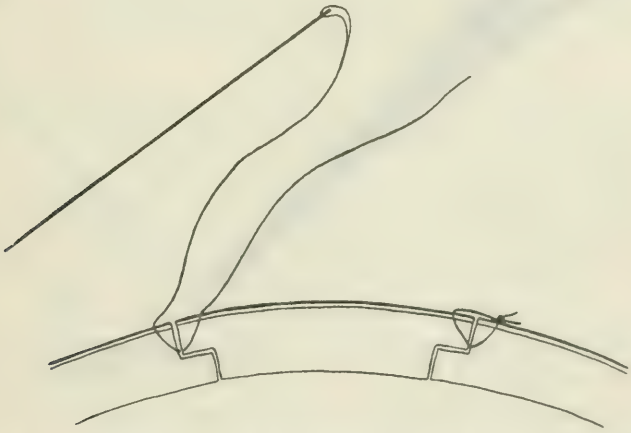
3. Resection of the Cornea and Transplantation.—The animal was etherized by the Meltzer-Auer method. The head was placed on a sand-bag and covered with a black silk towel, perforated in the center. The operating field was widely exposed by four small forceps fixed to the conjunctiva. The graft was placed on the surface of the cornea and the outline of the fragment to be resected was traced with the point of a cataract knife. Then the graft was replaced in olive oil. The incision of the cornea was made to a depth of about 1 mm. Then the internal edge was dissected for 1 mm., the anterior chamber was opened, and the fragment resected (Text-fig. 2). The graft was

immediately inserted in the opening, where it fitted the edges of the cornea exactly (Text-fig. 3).

4. Fixation of the Graft.—The suture of the graft to the cornea was made with straight needles, No. 16, and silk sterilized in vaseline. As the corneal tissue is very hard, prong-toothed dissecting forceps



TEXT-FIG. 2. Cross-section of corneal flap and opening in the cornea, showing step edges.

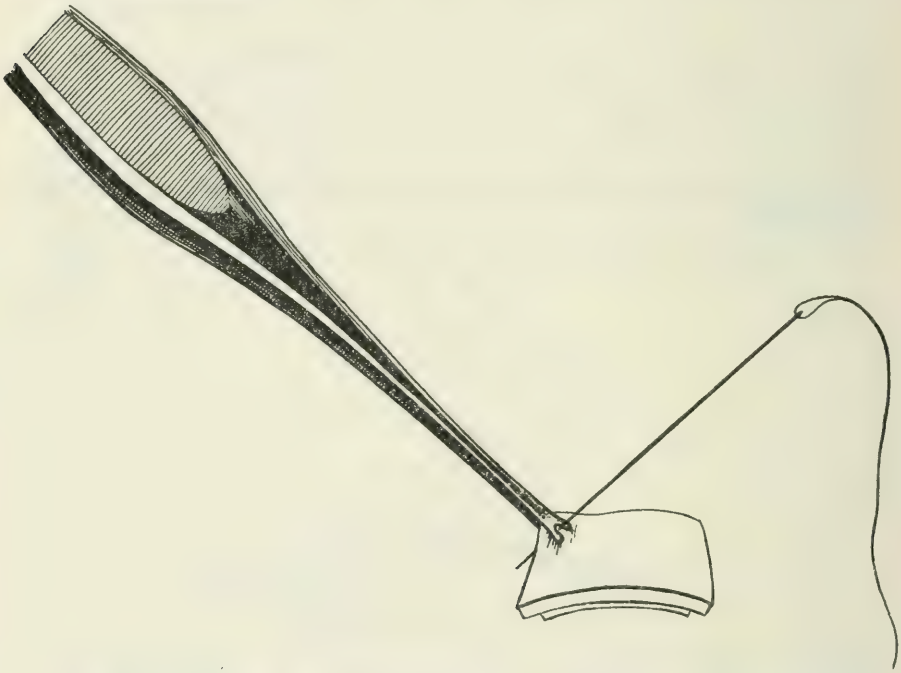


TEXT-FIG. 3. Transplanted flap in position, showing stitches passing through the superficial layers of the transplant and edge of opening in the cornea.

were used, the ends of which were bifurcated like a very small, double tined fork. The edge of the flap was seized by the forceps and the needle pushed through the tissue between the tines (Text-fig. 4). Only the superficial part of the cornea was caught by the needle. Each angle of the flap was fixed by one stitch. Another stitch was placed on the longer side of the rectangle. On account of the shape

of the edge, the incisions were closed tightly, and there was no leakage of fluid from the anterior chamber through the line of incision (Text-fig. 3).

5. Postoperative Treatment.—In previous experiments, it had been found that the dressing was often a cause of irritation. Therefore no dressing was applied. The animals kept the operated eye closed for several days after the transplantation. The corneal stitches fell out after a short time.



TEXT-FIG. 4. Prong-toothed dissecting forceps holding corneal flap and needle stuck through the tissue.

The experiments were made during an epidemic of distemper. Five cats were operated upon. Two died from distemper some time after the operations. Two others had a marked local infection of the eye and the cornea became completely opaque. In the fifth experiment (Cat 1, performed on May 20, 1919) the animal remained in good condition and the cornea transparent. A month later, the stitches had disappeared, the cicatrization was perfect, the cornea clear, and its curvatures did not appear to be modified. The iris was

not adherent to the cornea. There was a very small opaque spot near the inner canthus. Since that time, the animal has continued in the same condition. On May 26, 1921, the cornea was perfectly transparent, the only evidence of previous operation being a hardly visible opaque spot on the internal part of the cornea (Fig. 1). An examination was made by Dr. W. B. Doherty, the results of which are as follows:

No line of demarcation to indicate the limitation of the corneal graft could be seen with a Zeiss loupe. Corneal epithelium prefectly even. No lack of corneal luster. Oblique illumination showed no inequalities of the corneal surface. Cornea was sensitive. Transplant perfectly transparent; no infiltration or vascularization. Ophthalmoscopic examination showed clear image of the fundus. Near the inner canthus and in the portion comprising the original cornea, there was a faint infiltration of the deeper layers with two or three small blood vessels. This opacity was of a striate appearance and gave the impression that it was produced by trauma during the operative procedure.

The result obtained in this experiment shows that the technique which was used allows a perfect reconstruction of the cornea, even when a very large fragment has been resected. The cutting of the flap is not difficult after a little training, and there is no doubt that the fixation of a flap fitted like the door of a safe to the step-edged cornea by suture increased the safety of the operation in a great measure. It is known that, in the previous attempts to transplant the entire thickness of the cornea, a disc of cornea was merely placed in an opening made with a trephine. In the operation performed by Zirm, the disc was maintained by two cross threads stitched to the conjunctiva. There is certainly a great advantage in cutting and fixing the flap in such a way that the anterior chamber is tightly closed, and that no displacement of the graft can take place.

SUMMARY.

1. A flap composed of the entire thickness of the cornea of a cat was transplanted to the cornea of another cat, and was found to be perfectly transparent 2 years after the operation.

2. The curvatures of the cornea appeared to be normal.

We wish to acknowledge our indebtedness to Dr. Doherty for the clinical examination of the eye and the report of the findings.

EXPLANATION OF PLATE 34.

FIG. 1. Cat 1. Right eye operated upon. Photograph taken May 31, 1921, 2 years and 11 days after the transplantation was made. The cornea appears normal. There is practically no difference between the cornea of the left eye and that of the right.

FIG. 1.

(Ebeling and Carrel: Complete homotransplantation of cornea.)

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REMOTE RESULTS OF OPERATIONS ON THE PULMONARY ORIFICE OF THE HEART.

By ALEXIS CARREL, M. D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 6, 1921.)

In 1913, an attempt was made to develop a technique by which the size of the pulmonary orifice of the heart could be increased or decreased. The immediate results of the experiments showed that plastic operations on the wall of the pulmonary artery and its sigmoid valves caused little danger to the life of the animal, when a proper procedure was employed. The purpose of this paper is merely to examine the remote effects of the operations.

I.

EXPERIMENTAL.

In four experiments, the pulmonary orifice was enlarged by a vertical incision and suture of a flap of vessel.[1,2] In five others, the sigmoid valves were cauterized, incised, sutured, and the circumference of the orifice was shortened by stitching two of the valves together.[2,3,4]

1. Patching of the Pulmonary Orifice.—The operation consisted in suturing to the anterior side of the orifice a flap of a vein which permitted an increase in the circumference of the orifice after the wall had been incised.[1,2]

Experiment 1.—Black, female dog; medium sized, No. 1. October 23, 1913. The patch was a fragment of human aorta taken 24 hours previously from a cadaver and preserved in cold storage. The incision was made on the anterior side of the pulmonary artery. May, 1914. Animal still in normal condition. October 16. Died.

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[1] Tuffier, T., and Carrel, A., *J. Exp. Med.*, 1914, xx, 3.

[2] All operations were performed under ether anesthesia.

[3] Carrel, A., *J. Exp. Med.*, 1914, xx, 9.

[4] Carrel, A., *Ann. Surg.*, 1914, lx, 1.

Autopsy.—Adhesions of the lungs to the thoracic wall. Heart surrounded by adhesions. Adhesions of the pericardium. Anterior side of the pulmonary artery covered by a layer of fibrous tissue. Surface of the intima of the pulmonary artery smooth, and sigmoid valves entirely normal. Above the anterior valve, depression of the wall about 8 mm. wide, 18 mm. long, and 2 mm. deep.

Experiment 2.—Black and white bulldog, medium sized, No. 2. October 31, 1913. The patch was made of a flap of dog jugular vein, preserved for 24 hours in cold storage. May 20, 1914. Animal normal. March 3, 1915. Died after an illness of several weeks.

Autopsy.—Adhesions of the lung to the thoracic wall. Fluid in the pleural cavity. Adhesions of the pericardium. A thick layer of fibrous tissue on the anterior part of the pulmonary artery and the upper part of the ventricle. Indistinct and narrow scar above the anterior sigmoid valve of the pulmonary artery. No apparent depression of the wall. Surface of the intima smooth. Sigmoid valves normal. Location of the patch detected by thickening of the wall.

Experiment 3.—White, male bulldog, No. 3. November 26, 1913. Patching of the pulmonary artery with a piece of dog vena cava, preserved for 1 month in cold storage. May, 1914. Animal normal. February 17, 1915. Died.

Autopsy.—No adhesions of the pericardium. No adhesions of the pulmonary artery to the aorta or the auricle. Connective tissue on the anterior part of the vessel thickened. No scar visible from the outside. Opening of the vessel along the posterior side. Sigmoid valves normal. No apparent insufficiency. Slight depression of the wall above the anterior valve. Surface smooth and scar very indistinct. Upper part of the scar 1.5 cm. above the sigmoid and hardly visible. Location of the patch detected only by a slight thickening of the wall.

Experiment 4.—White, female bulldog, No. 4. December 2, 1913. The incision of the pulmonary artery was patched by a flap of jugular vein, preserved in cold storage since November 28, 1913. May, 1914. Animal in good condition. December 21. Died.

Autopsy.—No adhesions of the pleura or the pericardium. However, a few loose adhesions at the posterior part of the heart. Anterior part of the pulmonary artery free from fibrous tissue. No scar on the external side. After opening of the artery, an indistinct and slightly depressed scar visible above the anterior sigmoid. Surface of the intima smooth. Patch located by a slight thickening of the wall.

2. Cauterization, Section, and Suture of the Sigmoid Valves.—After longitudinal opening of the artery, just above the orifice, the sigmoid valves were cauterized with the thermocautery, or they were severed and sutured, or two of them were stitched together.[2,3,4]

Experiment 5. Cauterization of the Sigmoid Valves.—Young brindle, female bulldog, No. 5. April 14, 1914. Cauterization of the edges of the left and right

sigmoid valves with the thermocautery. June, 1921. Dog in good condition. No murmur.

Experiment 6. Cauterization of the Sigmoid Valves.—White and yellow, male fox-terrier, No. 6. April 29, 1914. Cauterization of the anterior and the right valves. December 29, 1916. Died.

Autopsy.—Adhesions of the lungs to the pericardium. Heart enlarged. Adhesions of the pericardium and the pulmonary artery to the aorta and the left auricle. Fibrous tissue on anterior face of the pulmonary artery. Anterior sigmoid valve rigid and thickened, especially along its margin. Left posterior valve normal. Two holes about the size of a pin-head near the anterior insertion of the right valve. Scar of the incision of the arterial wall hardly visible, especially in the center.

Experiment 7. Cauterization of the Sigmoid Valves.—White fox-terrier, with brindle spots, No. 7. April 30, 1914. Cauterization of the posterior valves. Animal remained in good condition for 6 years and died of double pleuropneumonia, May 31, 1920.

Autopsy.—No adhesions of the lungs to the thoracic wall. Adhesions of the lungs to the pericardium. Pus in both pleural cavities. Loose adhesions of the pericardium at the anterior part of the heart. Linear scar at site of pulmonary incision. Sigmoid valves normal; no thickening of the edges. A few small holes in the wall of the posterior sigmoids near the margin.

Experiment 8. Section of a Sigmoid Valve.—Black, long haired dog, No. 8. March 10, 1914. Section of the right posterior valve with the scissors. Animal died during the period of the War. Heart, preserved in formaldehyde, examined May, 1920. Adhesions of the pericardium. Valve slightly rigid. No union of the incision; thickening of the edges.

Experiment 9. Stenosis of the Orifice by Stitching Both Posterior Valves.—Black and white, long haired, male dog, No. 9. March 12, 1914. The right and left sigmoid valves were united by a stitch at a distance of about 3 mm. from their insertion. Dog remained in good condition. May 10, 1918. Died of pneumonia.

Autopsy.—Pericardial adhesions. Margin of both posterior valves slightly thickened. Scar tissue on the wall of the artery at the insertion of the valves. No union of the valves and, therefore, no stenosis of the orifice.

Experiment 10. Section and Suture of the Right Sigmoid Valve.—Yellow and white, male fox-terrier, about 7 years old, No. 10. March 17, 1914. Right sigmoid valve was completely sectioned in the middle from the margin to the insertion. Approximation of the cut edges by a stitch. May 20. Slight diastolic murmur. June 8, 1920. Animal in good condition. No murmur. June, 1921. Same condition.

II.

DISCUSSION AND SUMMARY.

Eight animals died from 1 to 6 years after the operation, from undetermined diseases, or from pneumonia. Two animals are still alive 7 years after the operation.

1. Condition of the Pleural and Pericardial Cavities.—In the first experiments, extensive pleural and pericardial adhesions were observed 1 year and more after the patching of the pulmonary artery. There was also a great deal of fibrous tissue between the pulmonary artery, the aorta, and the left auricle. In the other experiments, the adhesions of the lungs, pleura, and pericardium were less marked. This was due to some improvements in the technique of handling the viscera. At the time of the operations, it was hoped that no pleural or pericardial adhesions would occur. Great care was taken not to injure the endothelial surfaces by rough handling or by sponging. No blood was allowed to flow into the pleural cavity. The surface of the pericardium was protected by fine silk membranes. The pleural cavity was occluded by thick pads made of cotton and Japanese silk. It seemed that the serous surfaces were almost completely protected against infection and mechanical irritation. The occurrence of primary pleurisy and pericarditis was prevented by this technique. But the development of adhesions in several of the experiments shows that the procedures for the handling of the viscera should be perfected.

2. Condition of the Arterial Wall.—In the experiments in which the orifice was patched, a slight dilatation of the artery was observed. It was not possible to ascertain from the specimen preserved in formaldehyde whether or not there was an insufficiency of the valves. It is probable that there was no leakage, as in none of these cases could any diastolic murmur be heard 6 months after the operation. The only animal which presented clinical evidence of pulmonary insufficiency died during the War. The normal condition of the pulmonary orifice was due to the incision which did not extend far enough on the ventricle, and to the power of redintegration possessed by an organ which is not diseased. The cicatrization of the grafted flap was excellent. Its outline could not be seen on the external side of the wall. Even after opening the artery, the transplant could not be

located easily. However, in Experiment 1 the anterior wall of the artery showed a depression about 7 or 8 mm. wide, 18 mm. long, and 2 or 3 mm. deep, behind and above the anterior valve. But the flap was made of human artery, and it is known that a heteroplastic graft always undergoes some dilatation. When transplants of dog tissue were used, no dilatation occurred and the location of the patch could hardly be detected. In Experiment 7, 6 years after the operation, the endothelial surface was smooth, glistening, and no scar could be seen. However, the upper and lower parts of the incision were marked by a slight depression of the wall. The presence of the patch was detected by a distinct thickening of the wall. Although the edges of the incision had not been sutured to the edges of the flap, the endothelial surface was quite smooth. A transverse section of the artery was made through the middle part of the flap in Experiment 4. It showed the width of the arterial opening and the way in which the transplant became adherent to the arterial wall. The examination of these four specimens demonstrated that, in spite of the unfavorable location of the graft, an excellent union had taken place. It showed that homoplastic or heteroplastic tissue can be transplanted onto the pulmonary artery as well as onto the smaller arteries. Where the arterial wall had simply been incised without interposition of a patch, a linear scar was always found. 6 years after the operation, the incision used in the course of an operation for cauterization of the sigmoid and sutured with heavy thread was transformed into a linear scar and the surface of the intima was quite smooth.

3. Condition of the Sigmoid Valves.—In three experiments, the sigmoid valves had been cauterized along their margin and their point of insertion in the artery. One of the animals was still living 7 years after the operation. There was no diastolic murmur. The other animals died 3 and 6 years after the operation. The valves were thin and transparent, and quite normal. However, one of the valves showed two holes, one near the base and the other near the margin.

The animal on which the section of the right posterior valve without suture was performed, died 2 or 3 years after the operation. The

edges of the incision had not united. They were thickened and the whole valve was rigid. The surface was rough and irregular.

No permanent result was obtained by the union of two sigmoid valves by a stitch. There was no stenosis of the orifice, and no union of the valves 4 years after the operation. The stitch had disappeared. There was some scar tissue at the common point of insertion of the posterior valves, which were more rigid and showed thickened edges. 7 years after the section and suture of a sigmoid had been performed, the animal was still living and in good health. No diastolic murmur could be detected.

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GROWTH OF FIBROBLASTS AND HYDROGEN ION CONCENTRATION OF THE MEDIUM.

By ALBERT FISCHER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 6, 1921.)

The purpose of the experiments described in this paper was to determine the rôle played by the hydrogen ion concentration of the medium with regard to the growth of a strain of fibroblasts cultivated *in vitro* for a long period of time. The fibroblasts used were derived from fresh embryonic chick heart, from 1 or 2 months old strains of connective tissue, and from a strain cultivated for 9 years.[1] The cultures were prepared according to a technique previously described.[2] The method used for determining the hydrogen ion concentration has been described recently by Felton.[3]

I.

Method of Obtaining Variations in the Hydrogen Ion Concentration of the Media.

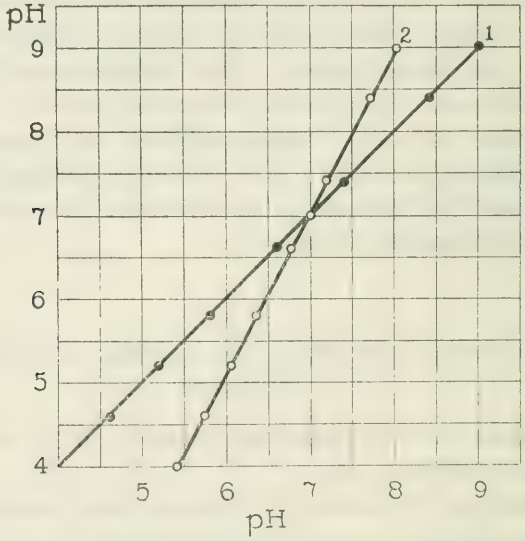
The variations in the hydrogen ion concentration of the media were obtained in the following ways.

1. Acid Solutions.—Solutions of acids were made in such concentrations that the addition of a small amount, usually 1 drop, to a certain amount of embryonic tissue juice or plasma would give the hydrogen ion concentration desired. A quantity of juice or plasma sufficient for a whole series of experiments was made in order to secure the same reaction in all. One of the disadvantages of this method was that plasma prepared in quantity underwent changes which resulted in decreasing or entirely preventing coagulation. A second disadvantage was that the embryonic tissue juice sometimes caused subsequent precipitation.

[1] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.
[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531–535.
[3] Felton, L. D., *J. Biol. Chem.*, 1921, xlvi, 299.

2. Phosphate Solutions.—Sörensen's standard phosphate solutions were added to embryonic tissue juice in equal parts, to obtain different hydrogen ion concentrations in the media. It was found that if equal parts of fresh embryonic tissue juice and sterile buffer solutions were mixed, for instance with a pH of 7, the growth obtained was nearly as extensive as that in the control in which the juice was diluted with Ringer solution. This showed that phosphate had no appreciable influence on the rate of growth.

A known hydrogen ion concentration was obtained by the following technique. Some preliminary experiments were made in order to find



TEXT-FIG. 1. The abscissæ indicate the pH obtained, and the ordinates the pH of the buffer solution added. Curve 1 represents the pH of Ringer solution, and Curve 2 the pH of embryonic tissue juice, to both of which are added the different standardized buffers as indicated by the ordinates.

out the pH of a mixture of embryonic juice and a given acid. Different numbers of drops of the respective solutions (acid or buffer) were added to tubes holding the same amounts of juice, and the hydrogen ion concentration was tested. These different known reactions of the juice obtained in this way gave a curve in which the amount of acid necessary to obtain a certain hydrogen ion concentration between the

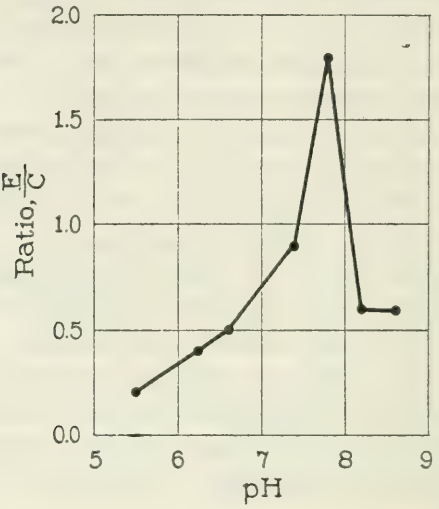
points found empirically could be calculated by interpolation (Text-fig. 1). Equal volumes of the standardized buffer solutions and of tissue juice were used. Upon ascertaining what pH resulted in the tissue juice when it was mixed with its own volume of buffer solution, the reactions obtained gave a straight line when a curve was plotted and it was possible to calculate readily which buffer should be used to produce the hydrogen ion concentration desired in the tissue juice (Text-fig. 1). Another test made in the same way was then necessary; namely, with the plasma-juice mixture. This had to be done rapidly in order to mix the indicator and juice-plasma before coagulation took place. In other words, when fibroblasts are to be cultivated in a medium of, let us say, pH 6, preliminary experiments for those particular media will show what buffer solutions should be added to the juice which will give pH 6, when combined with plasma. The test should be made in two steps for each experiment, one for the juice-buffer, and the other for the juice-buffer-plasma mixture, because of the slight differences in hydrogen ion concentration of the different juices and plasmas. The tissue juice may vary from pH 6.8 to 7.2, and the plasma from pH 7.4 to 8.

II.

Cultivation.

After the embryonic juice had been tested to determine its hydrogen ion concentration, it was mixed with equal parts of a buffer solution, calibrated pipettes being used to obtain the same sized drops. A selected culture was divided in two equal parts and washed in Ringer solution for about 30 seconds. One fragment was cultivated in a medium composed of 1 drop of plasma and 1 drop of a mixture of embryonic tissue juice and buffer solution. The other fragment, or the control, was cultivated in 1 drop of plasma and 1 drop of embryonic tissue juice to which had been added the same volume of Ringer solution instead of buffer solution. Mica cover-slips were used. 1 hour after the preparation of the culture, a drawing was made under the projectoscope. After 48 hours incubation, the outline of the new growth was drawn and calculations were made, by the method described by Ebeling.[2] When the purpose of the experiments was the study of the influence of the same hydrogen ion concentration for several genera-

tions, the culture was divided in two parts at each passage. One fragment was kept as control and the other placed in the experimental medium, care being taken to keep the other factors constant.



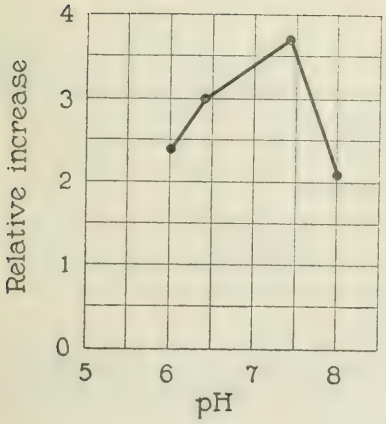
TEXT-FIG. 2. The different hydrogen ion concentrations of the media in which the fourth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth, $\frac{E}{C}$. The different hydrogen ion concentrations in the medium were obtained by adding phosphate buffer solutions to the extract.

III.

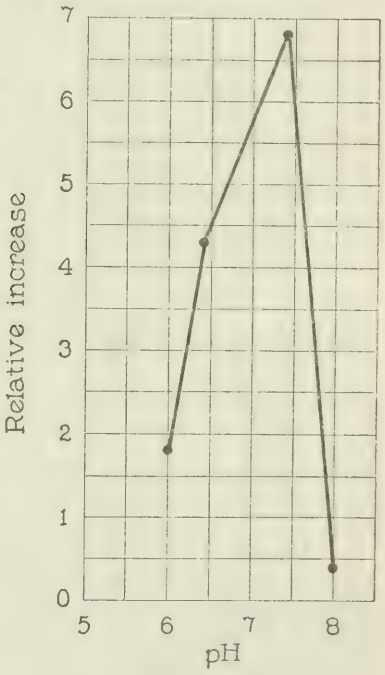
Rate of Growth.

When fibroblasts were cultivated in media containing different hydrogen ion concentrations varying from pH 5.5 to 8.5, a curve was obtained for the rate of growth with a distinct maximum between pH 7.4 and 7.8 (Text-figs. 2 to 4). The rate of growth decreased very rapidly with increasing hydrogen and hydroxyl ion concentrations, and the plotted curve was nearly symmetrical. The results obtained by using different acids, such as acetic, phosphoric, sulfuric, and hydrochloric, were much the same. It seemed that the anions did not have any remarkable influence on the growth. The curves reached a maximum at

pH 7.4 and fell very rapidly on both sides of this point, appearing always to be steeper on the alkaline side in spite of the fact that the resistance against the alkalinity was more marked. The same type of curve was found in all experiments. When the media were more alkaline or more acid, precipitation occurred, coagulation was interfered with, and it was impossible to draw any conclusions from the results. If the series of experiments were repeated by carrying the fibroblasts to a new medium containing the same hydrogen ion concentration as was used in the preliminary culture, it was observed that the absolute



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. The different hydrogen ion concentrations of the media in which the first passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TEXT-FIG. 4. The different hydrogen ion concentrations of the media in which the fifth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TABLE I.

Experiment No.	Culture No.	Buffer solution added.*	Juice-buffer mixture.	First passage.			Second passage.			Third passage.			Fourth passage.		
				Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.
		pH	*pH*												
1	264	4.0	5.5	7.0	0.4	0.6	6.2	3.1	0.5	3.0	2.7	0.9	6.1	1.7	0.2
2	265	4.6	6.0	5.4	0.1	0.1	6.3	2.4	0.3	6.6	3.0	0.4	6.5	0.9	0.1
3	266	5.2	6.2	3.1	7.3	0.4	16.5	4.7	0.2	3.0	1.7	0.5	6.6	3.0	0.4
4	267	5.8	6.6	1.8	1.5	0.7	8.7	4.1	0.4	4.6	3.3	0.7	3.0	1.7	0.5
5	268	6.6	7.2	4.7	3.9	0.8	12.4	1.8	0.1	4.2	5.1	1.2	5.8	3.4	0.5
6	269	7.0	7.4	10.8	10.8	1.0	12.2	11.9	0.9	4.8	6.8	1.4	7.3	6.6	0.9
7	270	7.4	7.8	10.4	6.6	0.6	4.3	5.1	1.1	9.9	12.1	1.2	2.1	3.9	1.8
8	271	8.4	8.2	11.8	4.5	0.3	12.1	7.9	0.6	10.3	8.1	0.7	3.7	2.3	0.6
9	272	9.0	8.6	7.2	6.3	0.8	11.1	7.7	0.7	12.7	8.4	0.6	7.6	4.9	0.6

* 1 cc. of buffer solution was added.

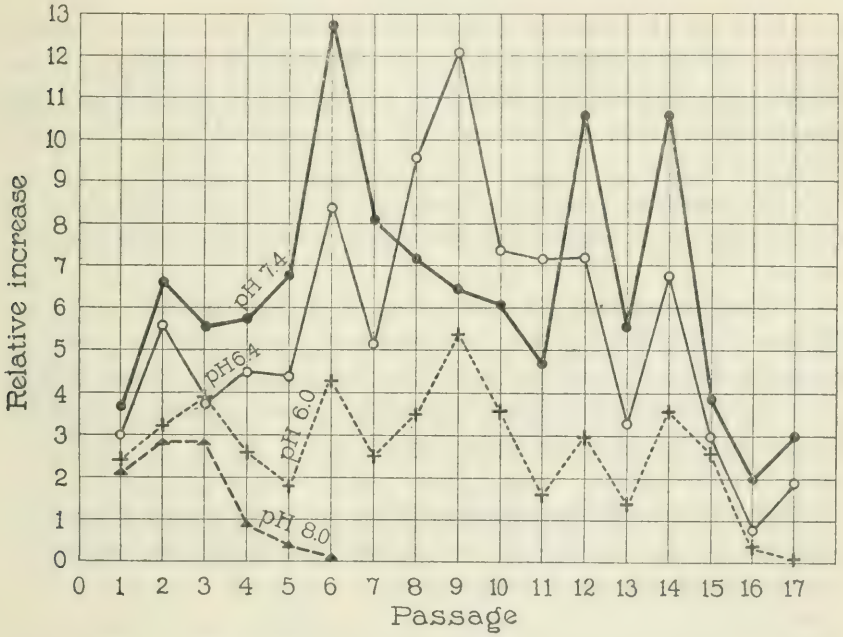
TABLE II.

Experiment No.	Culture No.	Buffer solution added.*	Juice-plasma mixture.	Relative increase. Passage No.																
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
		pH	*pH*																	
1	375–1	4.0	6.0	2.4	3.2	3.9	2.6	1.8	4.3	2.5	3.5	5.4	3.6	1.6	3.0	1.4	3.6	2.6	0.4	0
2	375–2	5.8	6.4	3.0	5.6	3.7	4.5	4.3	8.3	5.2	9.6	12.1	7.3	7.2	7.2	3.3	6.8	3.0	0.8	1.9
3	375–3	7.0	7.4	3.7	6.6	5.6	5.8	6.8	12.7	8.1	7.2	6.5	6.1	4.7	10.6	5.6	10.6	3.9	2.0	3.0
4	375–4	9.0	8.0	2.1	2.8	2.8	0.9	0.4	0	0	0	0	0	0	0	0	0	0	0	0

* 1 cc. of buffer solution was added.

increase of new growth became less and less in the most alkaline and most acid media (see also Tables I and II and Text-fig. 5). The descent of the curve on both sides of the maximum became more abrupt in the succeeding generations because of the retardation of growth in the media with highest alkalinity and acidity. (Compare Text-fig. 3, which represents the first passage, and Text-fig. 4, which represents

the fifth passage.) By following the growth of fibroblasts, generation after generation, in media containing the same hydrogen ion concentration it was observed that in the highest acidity (pH 5.5) cell proliferation ceased after four to six passages. The fibroblasts showed more resistance to higher alkalinity, but at the highest (pH 8.5) they grew for about eight to ten passages. It may be seen from the curve (Text-fig. 2) that the growth of tissue at pH 5.5 was very near the reaction where



TEXT-FIG. 5. The growth of the four cultures of the experiments in Table II, expressed graphically.

no growth will take place, although at pH 8.5 it was further from this point, and growth was observed for more than ten passages. It may be assumed, therefore, that growth can continue for a long time in this hydrogen ion concentration, but with a smaller increase of cell proliferation. The optimum growth occurred between pH 7 and 7.8. This was the normal reaction of a mixture of plasma and embryonic tissue juice. It may be seen from the curves that a slight variation in the hydrogen ion concentration of the culture medium resulted in marked changes in

the rate of growth of fibroblasts. It is interesting to note that the differences were mostly of a quantitative nature. The only morphological change of the cells observed was that, in the acid media, they showed more vacuoles than in the alkaline.

IV.

CONCLUSIONS.

1. The rate of growth of fibroblasts is markedly modified by slight changes in the hydrogen ion concentration of the medium. The curves expressing the rate of growth in function of the hydrogen ion concentration of the medium are nearly symmetrical on both sides of the maximum.

2. The optimum growth of fibroblasts occurs at pH 7.4 to 7.8. A slight change from this reaction has a remarkable influence on the rate of growth.

3. Fibroblasts show more resistance to higher alkalinity than to higher acidity. They grew for only four to six generations in a medium having a pH of 5.5, and for more than ten generations in one of 8.5.

4. The influence of different hydrogen ion concentrations on fibroblasts was only of a quantitative nature.

I wish to acknowledge my appreciation to Dr. Felton for his advice regarding his own method for measuring the hydrogen ion concentration of small amounts of fluid.

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COMPARATIVE HYDROLYSIS OF GELATIN BY PEPSIN, TRYPSIN, ACID, AND ALKALI.

By JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Gelatin and proteins in general may be hydrolyzed by either acids (hydrogen ions), alkali (hydroxyl ions), pepsin, or trypsin. In all cases the reaction consists in the rupturing of one or more of the peptide linkings with the addition of 1 molecule of water. The ease with which the various linkages are split is very different, as was shown by Fischer,[1] in his study of the hydrolysis of the polypeptids. The same phenomenon is shown by the fact that the products of partial hydrolysis of the proteins form a series of increasing complexity—a result of the fact that some of the linkages are much more easily split than others. The same fact is brought out by following the course of the reaction in strong acid for example. If all the linkages were split at the same rate, the reaction would follow the course of a monomolecular one. This is not the case. The velocity decreases steadily as the reaction proceeds, showing that some of the linkages are split more easily than others.

The question arises then as to whether those linkages which are most easily split by acid, for instance, are also the most easily split by pepsin and trypsin. There is evidence already that this is not the case. Henriques and Gjaldbäk[2] have shown by an ingenious modification of the formol titration that protein solutions having the same total content of titratable amino or carboxyl groups but which have been brought to this stage by different hydrolytic agents, may be distinguished from each other by their behavior when titrated with alkali to different end points, showing that, although the total

[1] Fischer, E., Untersuchungen über Aminosäuren Polypeptide und Proteine. Berlin, 1906.

[2] Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxv, 363; 1913, lxxxiii, 83.

number of linkages split was the same in all the solutions, different ones had been split in each case. They found that the course of the reaction was similar with pepsin and acid, and with trypsin and alkali. The study of the hydrolytic products of trypsin and pepsin leads to the same conclusion.[3] It is usually stated that trypsin hydrolysis leads to more amino-acid formation.

More quantitative evidence may be obtained, however, by a slightly different mode of procedure; namely, by adding the enzyme in question to a solution of the protein which has already been partially hydrolyzed by some other means and noting the subsequent increase in hydrolysis. If the two reactions follow the same course, *i.e.*, if the same linkages are split in both cases, the final amount of hydrolysis will be the same irrespective of the stage at which the enzyme is added. If, however, the preceding hydrolysis has split the protein at a linkage which is not attacked by the enzyme, then the total amount of hydrolysis will be the greater the farther the hydrolysis had proceeded before the enzyme was added. That is, the total hydrolysis will be equal to the sum of the enzyme hydrolysis (on the unhydrolyzed protein) plus that which had taken place due to the other hydrolytic agent. Since it is known that acids and alkali are capable of hydrolyzing the proteins to their constituent amino-acids, it is obvious that they must be able to split all the linkages so that a stage must eventually be reached in acid or alkali hydrolysis at which the addition of the enzymes will cause no further hydrolysis. The stage in the hydrolysis at which this occurs will evidently be a measure of the difference in the course of the two reactions. If the linkages which are most easily split by pepsin, for instance, are the ones which are most slowly attacked by acid, it will evidently be necessary to almost completely hydrolyze with acid the protein before reaching a stage at which the addition of pepsin will cause no further increase. As will be seen below, this is really the case.

The results are complicated by the fact that the ease of hydrolysis depends on other factors as well as on the particular linkage which is hydrolyzed. This was shown by Fischer,[1] who found that tetraglycyl glycine may be hydrolyzed by trypsin whereas triglycyl glycine is not

[3] *Cf.* Fischer.[1]

hydrolyzed. It is, therefore, not possible to conclude that the enzyme ceases to act, when a certain stage of the hydrolysis has been reached, because all the linkages which it is capable of hydrolyzing have already been split. The molecule may have been modified in some other way and so become resistant to the action of the enzyme although still containing a linkage which under certain conditions may be attacked. The same influence is undoubtedly also shown on the rate of hydrolysis by acids or alkali. There does not seem to be any evidence to distinguish qualitatively between the specificity of an enzyme and of hydrogen ions.

The procedure outlined above was followed in the experiments described in this paper. The gelatin was hydrolyzed to various stages by one of the hydrolyzing agents mentioned, pepsin or trypsin then added, and the increase in hydrolysis noted. The reaction was followed by means of a slight modification of the formol titration.

EXPERIMENTAL.

Gelatin.—Powdered gelatin, purified by washing at the isoelectric point,[4] was used in all the experiments. The solutions were made up to contain 5 gm. of gelatin per 100 cc.

Pepsin.—The pepsin used was an active preparation of Fairchild Bros. (U. S. P. 1/19,000). It was prepared for use by dissolving 5 gm. in 100 cc. of water, adjusting to pH 2.5 with HCl and dialyzing under pressure against 0.01 N HCl for several days. The solution thus obtained had a very low titration which remained constant. In the concentration used the correction for the pepsin solution was negligible. The solution is referred to as 5 per cent pepsin.

Trypsin.—Fairchild's trypsin was used. It was prepared for use by dissolving 10 gm. in 100 cc. of water and dialyzing under pressure against running tap water at 6°C. for about 18 hours. The solution is very unstable and loses its activity in 3 or 4 days even at 2°C. The formol titration of this solution was negligible in the concentrations used and remained constant. This solution is referred to as 10 per cent dialyzed trypsin.

Hydrogen ion determinations.—The determinations were made by the E. M. F. method.

Formol titration.—The titration was carried out as described in a previous paper.[5] The method consists essentially in freeing the solutions from CO_2 by adjusting the pH with acid, and boiling for a few seconds. The solution is then

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

[5] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 715.

accurately adjusted to pH 7.0, using neutral red as an indicator. The formaldehyde is then added and the titration carried out as usual with 0.1 N NaOH, using thymol blue as indicator and an end-point of about 8.2. The titration was made on 10 cc. until the titration figure became more than 5 cc., after which 5 cc. were analyzed. The results have been calculated on the basis of 10 cc. of 5 per cent gelatin. The titration value for this concentration of gelatin was found to be 2.0 cc. The complete hydrolysis of gelatin increases the amino nitrogen (or formol titration) about 20 times.[5,6] The percentage total hydrolysis may therefore be found from the figures by dividing by 40.0.

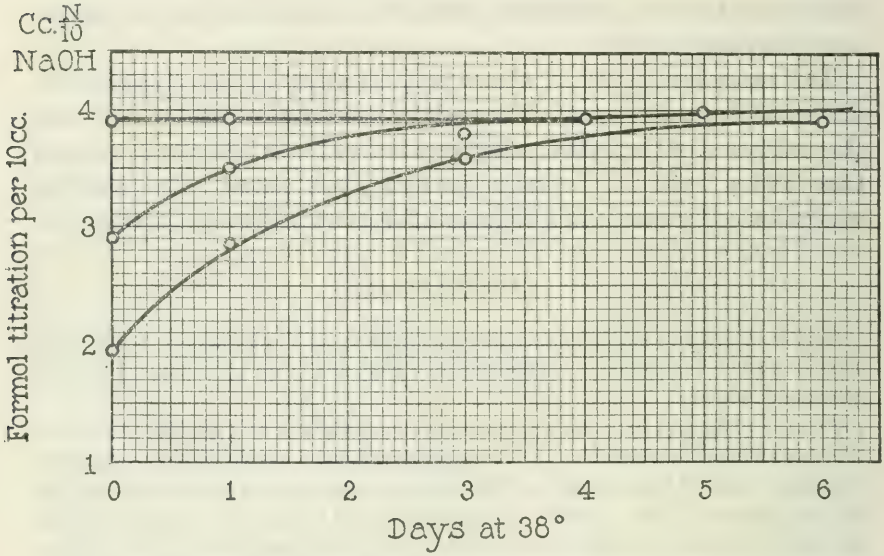

FIG. 1. Hydrolysis of gelatin by pepsin. 500 cc. of solution containing 25 gm. of gelatin, 20 cc. of 5 per cent dialyzed pepsin, and 40 cc. of 1.0 N HCl placed at 38°C. (pH = 2.2). 50 cc. samples pipetted out after 0, 1, and 4 days, and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin added to each sample and the samples placed at 38°C. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Hydrolysis by Pepsin Alone.

The results of this experiment are given in Fig. 1. The figure shows that under the conditions of this experiment, the pepsin is able to double the formol titration of the gelatin (an increase of 2 cc. per 10 cc. of 5 per cent gelatin); *i.e.*, the number of free carboxyl groups

[6]Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 49.

is doubled. Since in completely hydrolyzed gelatin the free carboxyl groups (or the amino nitrogen) is increased about twenty times, the hydrolysis due to pepsin alone corresponds to about 5 per cent of the total hydrolysis. It will be seen from the figure that this result is independent of the amount of pepsin added and also of the stage of hydrolysis at which the pepsin is added. It is not possible to say, however, whether or not this really represents the complete action of the pepsin, since the hydrolysis is still continuing slowly and if the analyses were made at weekly intervals it would be found that the values were increasing. This slow increase, however, approaches asymptotically the increase due to the acid alone, so that it is impossible to say when the action of the pepsin stops. The real end-point for pepsin digestion could be definitely fixed only by reaching the same value from both sides. For the purpose of these experiments it is permissible to take the end-point as that point at which the addition of more pepsin causes no further hydrolysis in the course of 3 or 4 days (the action of the acid alone in this time is within the limits of error of the method). In the experiment just described this value evidently corresponds to a titration of 4 cc. of 0.1 N NaOH per 10 cc. of 5 per cent gelatin.

Action of Pepsin on Gelatin Partially Hydrolyzed by Trypsin.

The results of this experiment are shown in Fig. 2. The experiment shows that the increased hydrolysis due to the pepsin becomes less the farther the hydrolysis due to the trypsin has been carried. The linkages which are split by pepsin are also evidently attacked by trypsin. At the same time, however, the trypsin is also hydrolyzing some linkages which are not attacked by pepsin. This is shown by the fact (Table I) that the addition of pepsin to a solution which has been hydrolyzed by trypsin to a titration of 7.0 cc. causes a still further increase, although, as we have seen, pepsin alone can only carry the hydrolysis to 4.0 cc. The same result was obtained by Henriques and Gjaldbäk[2] with other proteins.

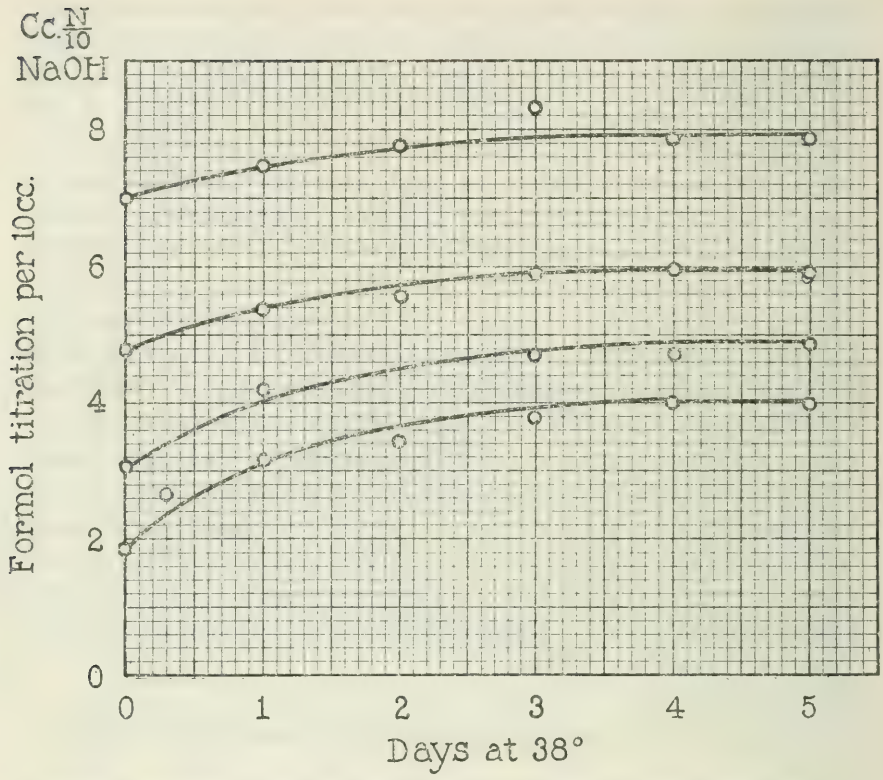

FIG. 2. Action of pepsin on gelatin previously partially hydrolyzed by trypsin. 500 cc. of 5 per cent gelatin containing 25 cc. of 1.0 M Na_2CO_3 placed at 38°C. and 5 cc. of 10 per cent dialyzed trypsin solution added (pH = 9.5). 25 cc. pipetted out at intervals and 2 cc. of 4.2 M HCl added (pH = 1.8), and the samples kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added to each sample and the samples replaced at 38° Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE I.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Trypsin.

	Formol Titration per 10 cc. of Gelatin Solution.			
Before addition of pepsin.....	1.9	3.1	4.8	7.0
Increase in titration due to action of pepsin..	2.1	1.9	1.1	0.9

Action of Pepsin on Gelatin Partially Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 3 and Table II. The results are very similar to those obtained with trypsin and pepsin. The linkages attacked by pepsin are evidently quite rapidly attacked by the alkali although some are still left after the hydrolysis has reached titration value of at least 10 cc.

Action of Pepsin on Gelatin Partially Hydrolyzed by Acid.

These experiments are summarized in Fig. 4 and Table III. They show that the linkages attacked by pepsin are among the most resistant to the action of acid since the addition of the pepsin caused the normal increase of 2 cc. even after the acid hydrolysis had proceeded to a value of over 6.0 cc. There is no evidence of a true equilibrium in the presence of pepsin since no *decrease* is noted at any time even when the acid hydrolysis has been carried far beyond the end-point reached with pepsin acting on the unhydrolyzed protein. Such a reverse or synthetic action of pepsin has occasionally been recorded (the so called plastein formation[7]) in the case of other proteins. It is apparently always connected with the formation of a precipitate. This fact makes it appear possible that the decrease in the titration value is due to the formation of some insoluble compound from substances present in the pepsin and protein solutions. This decrease has not been noted in the case of gelatin solutions and these are the ones in which no precipitate forms. The experiments described here show that the hydrolysis by pepsin follows quite a different course from that followed by acid hydrolysis. The products formed must therefore be different. It seems unlikely that the pepsin could have any synthetic action on a solution containing substances which differ from those formed by the action of pepsin itself.

Hydrolysis by Trypsin.

The extent of the hydrolysis by trypsin alone is shown in Fig. 5. The hydrolysis continues until a titration value of about 15 cc. is reached. The same uncertainty as to whether this is the real end-point evidently exists here just as in the pepsin hydrolysis.

[7] Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxi, 485; 1912, lxxxi, 439.

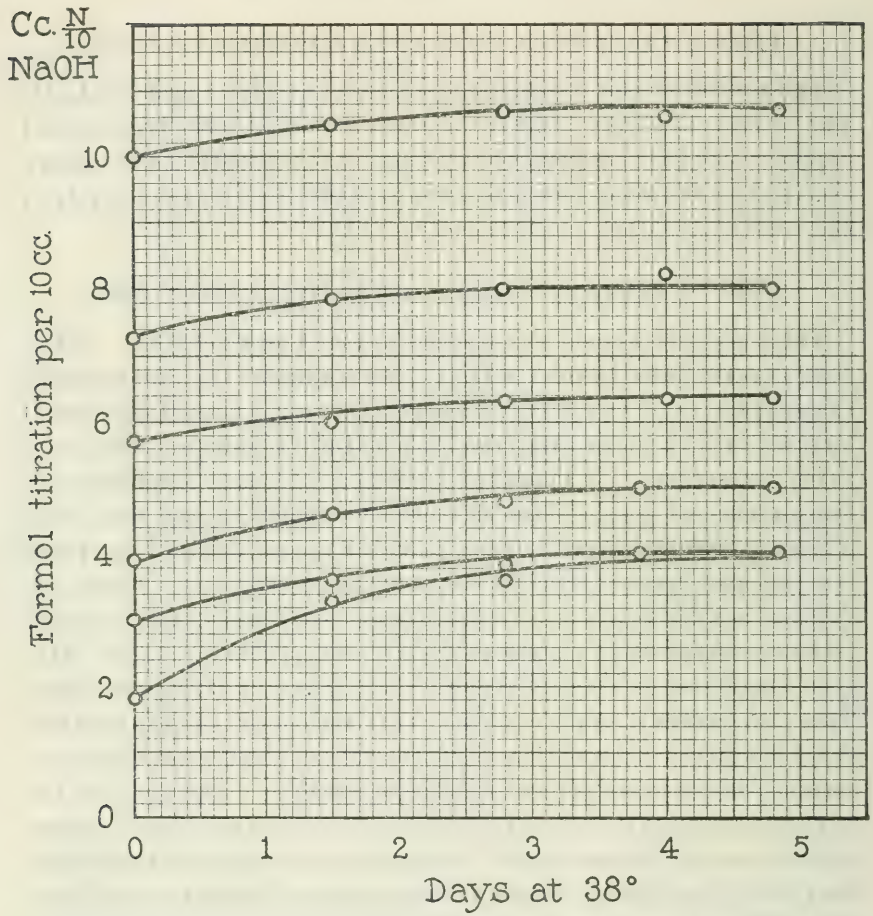

FIG. 3. Action of pepsin on gelatin partially hydrolyzed by alkali. 500 cc. of 10 per cent gelatin containing 8 cc. of 3 N NaOH kept at about 80°C. 50 cc. pipetted out at intervals and added to 50 cc. of 0.3 N HCl (pH = 2.0), these samples kept at 2° until all had been taken and 5 cc. of 5 per cent pepsin then added to each, and the samples placed at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE II.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Alkali.

	Formol Titration per 10 cc. of Solution.					
Before addition of pepsin	1.9	3.0	3.9	5.7	7.3	10.0
Increase due to action of pepsin	2.0	1.0	1.1	0.7	0.7	0.6

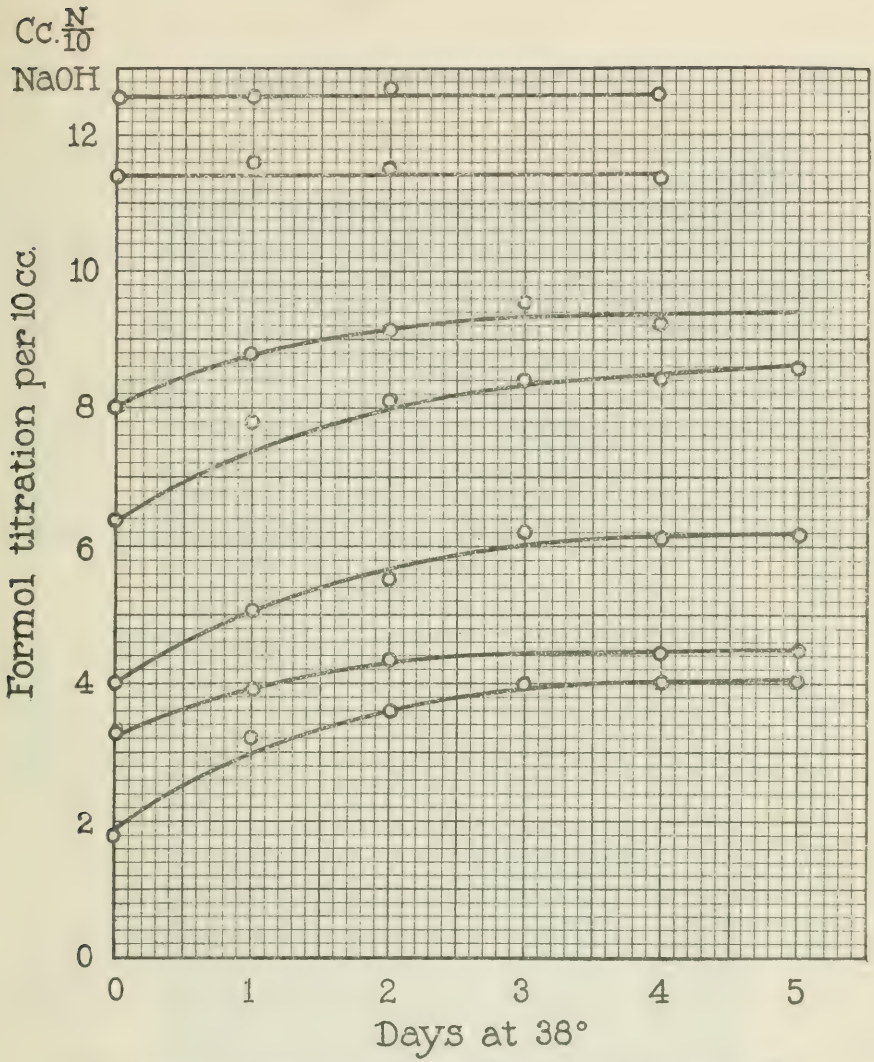

FIG. 4. Action of pepsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin containing 12 cc. of 4.0 M HCl (pH 1.8) kept at 38°. pH kept constant by addition of more HCl from time to time. 50 cc. samples pipetted out at intervals of 2 days and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added and the samples replaced at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE III.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Acid.

	Formol Titration per 10 cc. of Solution.						
Before addition of pepsin	1.9	3.3	4.0	6.4	8.0	11.4	12.6
Increase due to pepsin	2.1	(1.2)	2.0	2.0	1.0	0.0	0.0

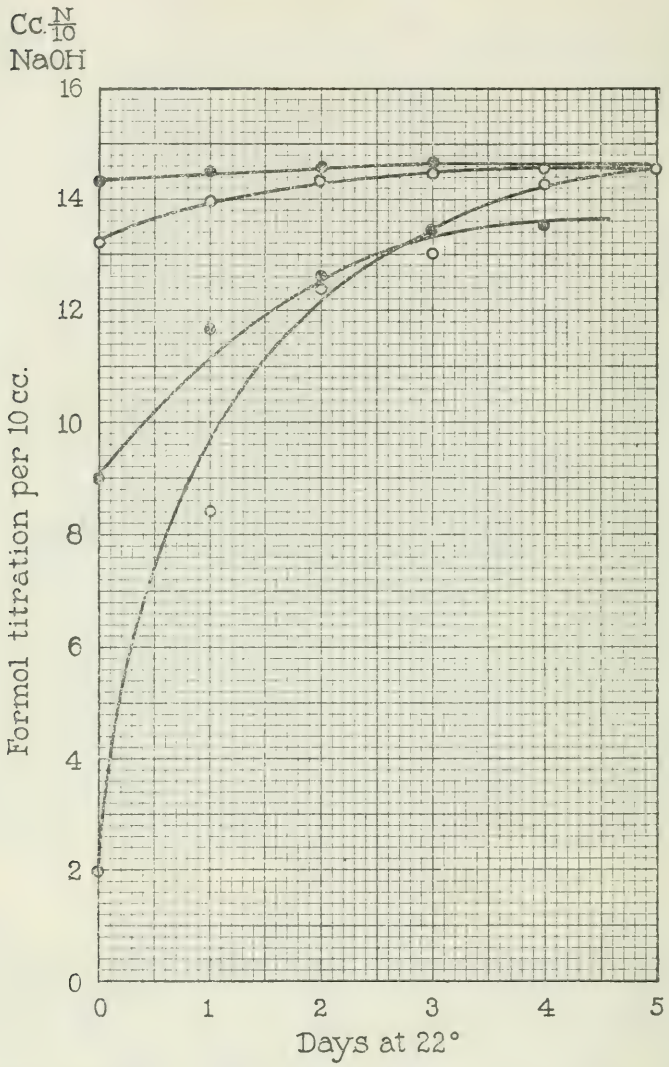

FIG. 5. Hydrolysis of gelatin by trypsin alone. 8 cc. of 10 per cent trypsin were added to 200 cc. of 5 per cent gelatin containing 10 cc. of 1.0 M Na_2CO_3, and the solution kept at 22°. 25 cc. samples pipetted out at intervals and kept at 3°C. until all had been collected. 1 cc. of 10 per cent trypsin was then added to each sample, the bottles placed at 22°, and formol titration was run on 5 cc., as shown in the figure. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Action of Trypsin on Gelatin Partially Hydrolyzed by Pepsin.

The results of this experiment are shown in Fig. 6. The final point reached by the trypsin hydrolysis is independent of the amount of hydrolysis previously accomplished by the pepsin. The linkages hydrolyzed by pepsin are therefore evidently also hydrolyzed by trypsin so that it is immaterial whether the pepsin acts on the protein or not, as far as the final stage reached is concerned. It is possible that the *rate* of hydrolysis may be greater if the pepsin acts first since Fig. 2 showed that those linkages attacked by pepsin are among the most resistant to the action of trypsin. The experiment confirms the result shown in Fig. 2, that trypsin hydrolyzes the same linkages as does pepsin but also attacks others which are split slowly if at all by pepsin.

TABLE IV.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Alkali.

	Formol Titration per 10 cc. of Solution.			
Before addition of trypsin	2.0	4.9	9.6	13.0
Increase due to trypsin	8.6	4.8	0.7	0.0

TABLE V.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Acid.

	Formol Titration per 10 cc. of Gelatin.			
Before addition of trypsin	2.0	6.7	10.2	18.0
Increase due to trypsin	8.2	4.5	5.4	5.0

Action of Trypsin on Gelatin Previously Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 7 and Table IV. Alkali hydrolysis evidently follows very nearly the same course as does trypsin hydrolysis since the increased hydrolysis due to the addition of trypsin becomes rapidly less and stops when the alkali hydrolysis has reached about 13 cc. This corresponds to the point reached by trypsin alone. The linkages split must therefore be the same in both cases. In this case also no evidence of a reversal of the action was observed although the experiment was carried much further than is shown in the figure.

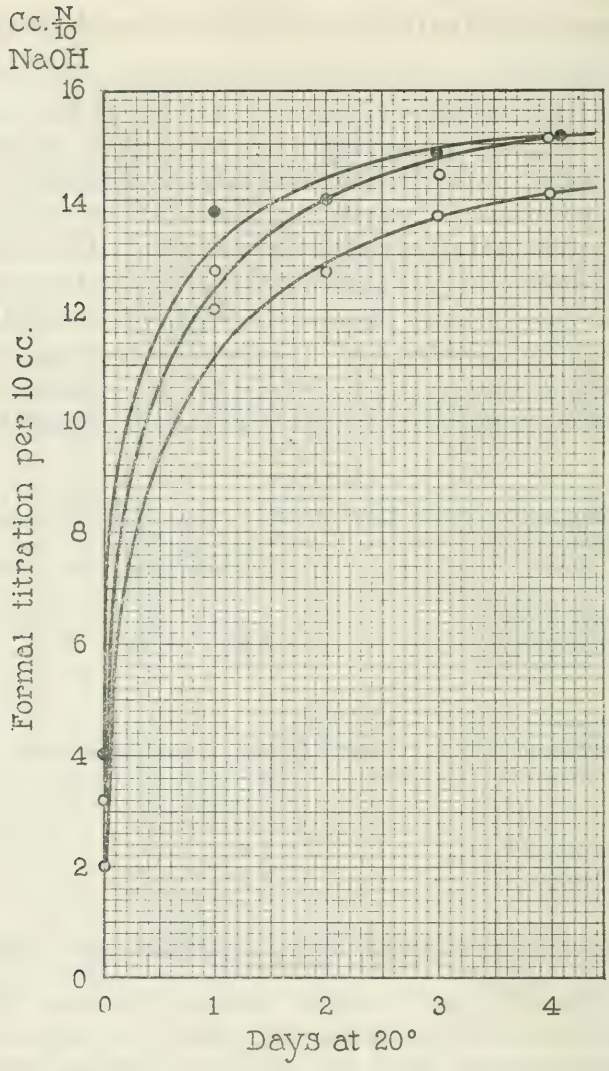

FIG. 6. Action of trypsin on gelatin partially hydrolyzed by pepsin. 500 cc. of 5 per cent gelatin solution brought to pH 2.2 with HCl, 20 cc. of 5 per cent pepsin solution added and the solution kept at 38°C. 25 cc. samples pipetted out at intervals, 5 cc. of 1.0 M Na_2CO_3 added and the samples kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added and the samples placed at 22°. Formol titration was run on 5 cc. samples. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

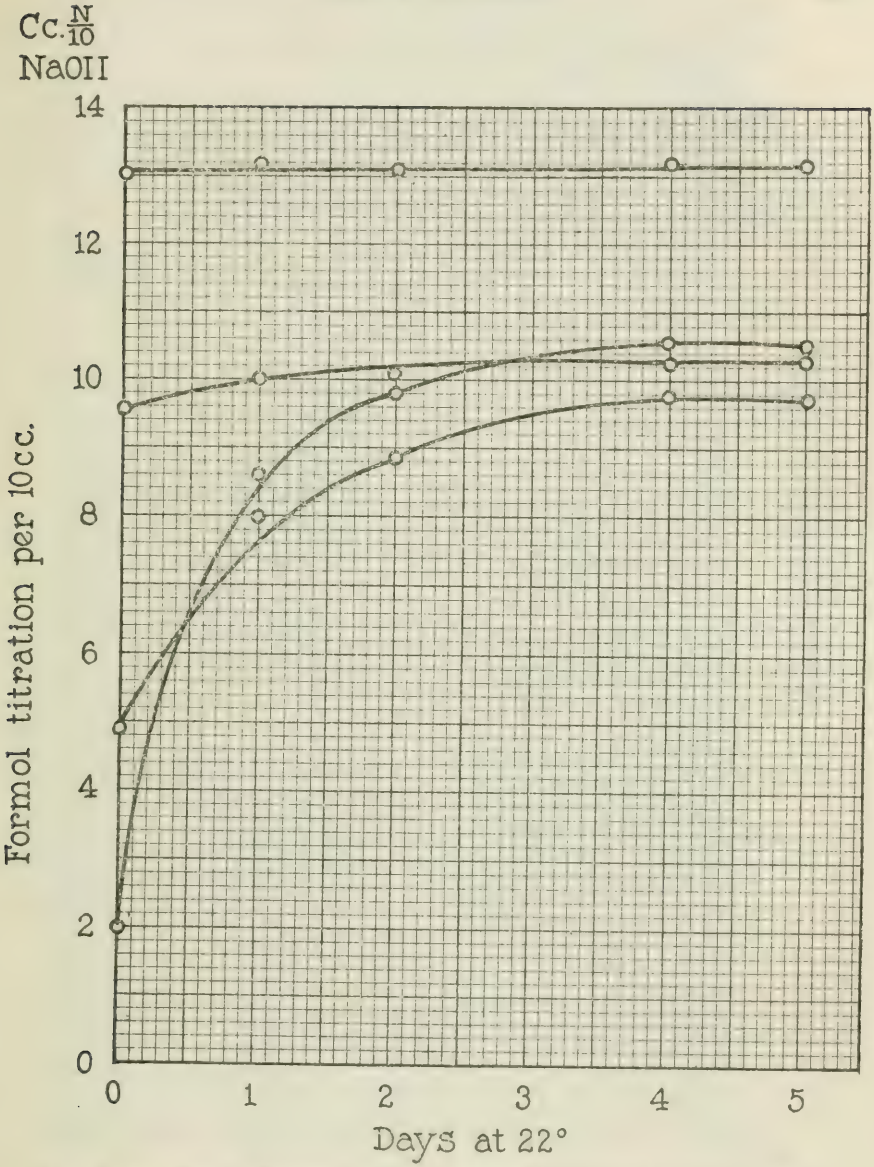

FIG. 7. Action of trypsin on gelatin partially hydrolyzed by alkali. 500 cc. of 5 per cent gelatin solution containing 50 cc. of 1.0 M Na_2CO_3 kept at 90°C. 25 cc. samples pipetted out at intervals, and kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added to each sample and the solutions kept at 22°. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

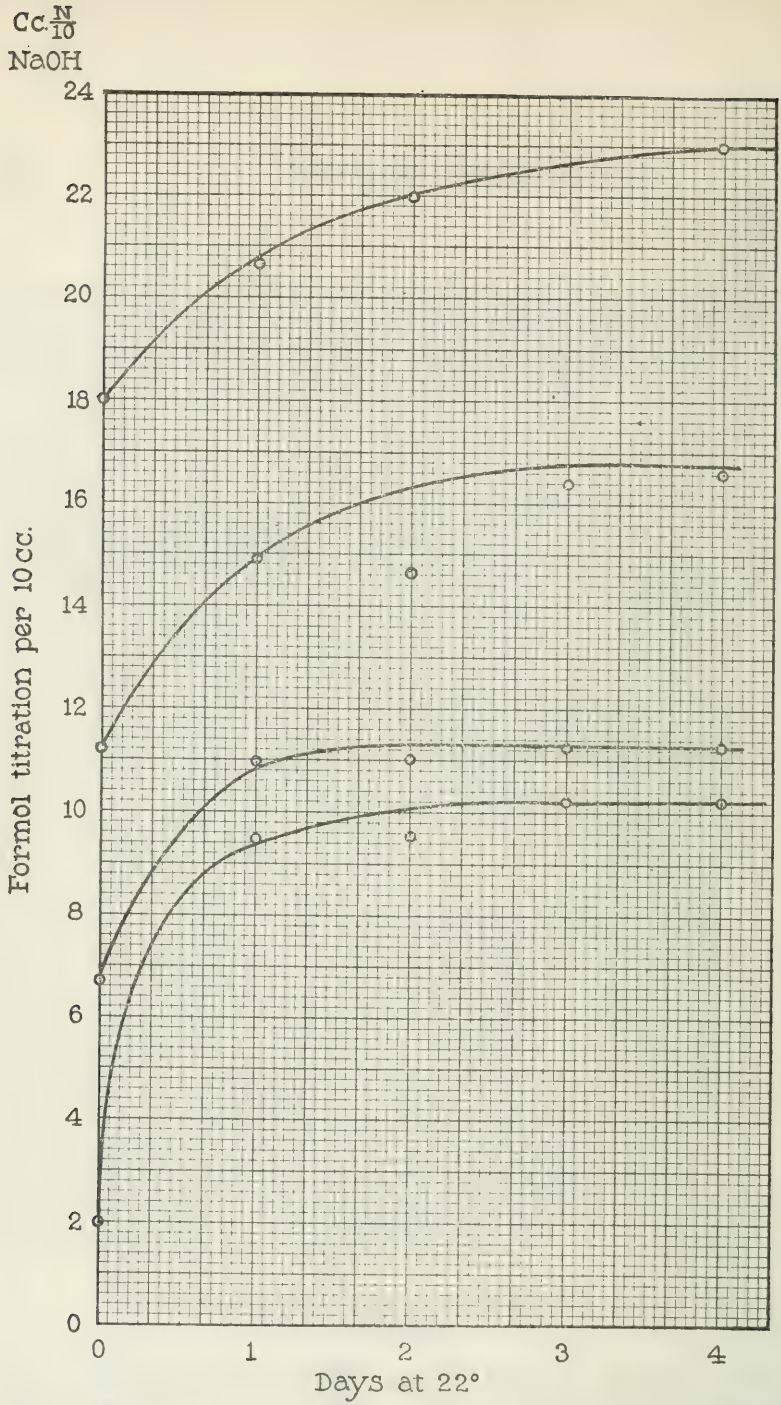

FIG. 8. Action of trypsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin brought to pH 1.8 with HCl and the solution kept at 90°C. 25 cc. samples pipetted out at intervals and 5 cc. of 1.0 M Na_2CO_3 added to each. Samples kept at 3° until all had been collected. They were then put at 22° and 1 cc. of 10 per cent dialyzed trypsin added to each sample. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Action of Trypsin on Gelatin previously Hydrolyzed by Acid.

The results of this experiment are shown in Fig. 8, and Table V. The course of the acid hydrolysis is quite different from that due to the trypsin since the addition of trypsin to the hydrolyzed gelatin is able to cause a still further increase even after the total number of linkages already split by the acid is greater than that which could be split by trypsin alone.

SUMMARY.

A comparison has been made of the relative velocity of hydrolysis of the various peptid linkings of the gelatin molecule when hydrolyzed by acid, alkali, pepsin or trypsin. It has been found that:

1. Those linkages which are most rapidly split by pepsin or trypsin are among the more resistant to acid hydrolysis.
2. Those linkages which are hydrolyzed by pepsin are also hydrolyzed by trypsin.
3. Trypsin hydrolyzes linkages which are not attacked by pepsin.
4. Of the linkages which are hydrolyzed by both enzymes, those which are most rapidly hydrolyzed by pepsin are only slowly attacked by trypsin.
5. Those linkages which are attacked by trypsin or pepsin are among the ones first (most rapidly) hydrolyzed by alkali.

In general it may be said that the course of the early stages of hydrolysis of gelatin is similar with alkali, trypsin, or pepsin and quite different with acid.

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DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

IV. VISCOSITY—*Continued.*

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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1. In order to complete the demonstration that the viscosity of suspensions of powdered gelatin in water is influenced by electrolytes in a similar way as the viscosity of solutions of gelatin, it is necessary to prove that the characteristic valency effect exists also in the case of the viscosity of suspended particles of solid gelatin in water.

This proof is furnished in Fig. 1. 0.5 gm. of finely powdered gelatin (going through a sieve of mesh size 100 but not through a sieve of mesh size 120) of pH 7.0 was put into a series of beakers containing each 100 cc. of HCl of different pH and kept in the solution over night at a temperature of 20°C. Simultaneously a similar series of beakers containing each 100 cc. of H_3PO_4 and H_2SO_4 of different pH (instead of HCl) were prepared, each receiving also 0.5 gm. of powdered gelatin. After 19 hours the viscosities of all these series of suspensions were determined at 20°C. Fig. 1 gives the result, the ordinates being the values for the viscosity ratios, gelatin suspension: water, and the abscissæ are the pH of the solid gelatin particles at equilibrium. The curves show that the viscosity of suspensions of gelatin sulfate is a little less than half that of suspensions of gelatin chloride and phosphate of the same pH. The curves for the suspensions of gelatin chloride and gelatin phosphate are alike, with the exception of part of the descending branch.

Experiments on the influence of these three acids on swelling published in a previous paper[1] show that the curves for the relative volume of powdered gelatin in solutions of these three acids are similar to the

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 253.

viscosity curves in Fig. 1 of this paper since the relative volume of gelatin sulfate was found to be not far from one-half of that of gelatin chloride or gelatin phosphate of the same pH. This demonstration completes the proof that the viscosity of *suspensions* of powdered gelatin in water of different pH is influenced in the same way by

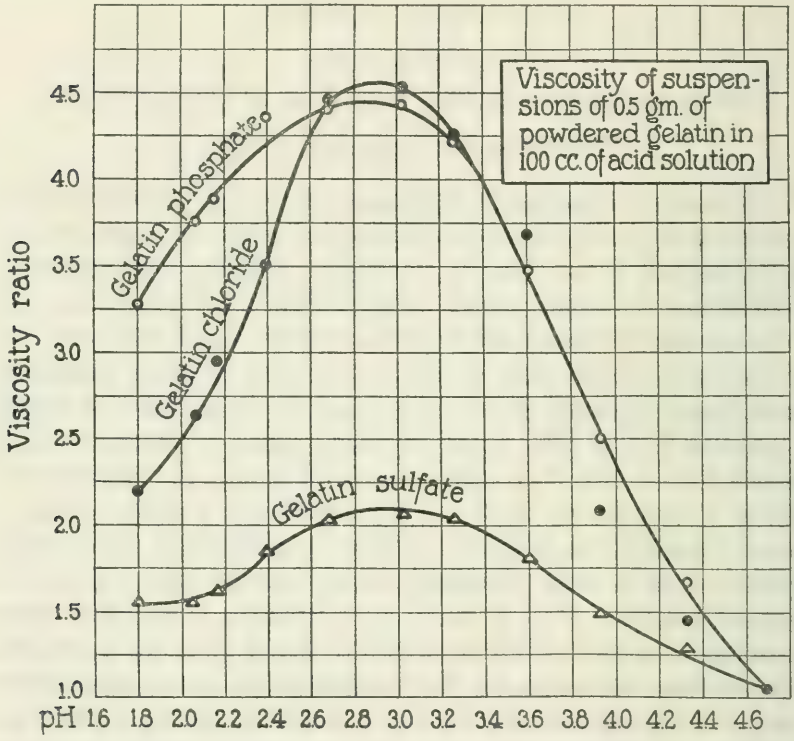

FIG. 1. Viscosity of suspensions of 0.5 gm. of powdered gelatin of grain size 100 to 120. Abscissæ are the pH, the ordinates the ratio of time of outflow of suspension: time of outflow of water. The influence of HCl and H_3PO_4 is practically identical for the same pH while H_2SO_4 depresses the viscosity of the suspensions to a little less than one-half of that for HCl.

electrolytes as is the viscosity of *solutions* of the same gelatin salts, and that this influence is due, in the case of suspensions, to the influence of the Donnan equilibrium upon the swelling of the particles. The volume V of gelatin occupied in 100 cc. of the suspension was determined by filtering and deducting the volume of the filtrate

from the total volume of the suspension. Knowing the viscosity we can calculate Einstein's constant c according to the formula

$$\left(\frac{\eta}{\eta_0} - 1\right)\frac{100}{V} = c$$

where c should be 2.5 if V is sufficiently small.

The values in Table I show that Einstein's formula gives the correct values for viscosity when the volume, V, of the gelatin is small, since in that case c is equal, or nearly equal, to 2.5, as his formula demands.

When, however, the volume is larger, the value for c exceeds 2.5. The fact that the value for c exceeds 2.5 when the relative volume occupied by the particles in the solution is large, was found also by

TABLE I.

V	$\frac{\eta}{\eta_0}$	c
cc.		
12	1.292	2.5
17	1.480	2.8
18	1.792	4.4
20.5	2.064	5.1
20.5	2.020	4.9
20	1.855	4.2
18	1.625	3.5
16.5	1.542	3.3

Hatschek,[2] Smoluchowski,[3] and Arrhenius.[4] Hatschek replaced the value 2.5 in Einstein's formula by a larger one, namely 4.5. This, however, meets in our case with the difficulty that the value c shows a drift reaching a maximum when the volume of the gelatin particles is a maximum. This difficulty is largely avoided in Arrhenius's formula and we have to change from Einstein's formula to that of Arrhenius whenever the relative volume of the particles in solution or suspension exceeds the limits of the applicability of Einstein's formula, as we shall see in the next chapter.

[2] Hatschek, E., *Kolloid Z.*, 1913, xii, 238; 1920, xxvii, 163.

[3] Smoluchowksi, M., v., *Kolloid Z.*, 1916, xviii, 190.

[4] Arrhenius, S., *Meddelanden from K. Vetenskapsakademiens Nobelinstitut.* 1917, iii, No. 21.

The experiments on the viscosity of suspensions of powdered gelatin in water have, therefore, led to the result, first, that the influence of pH, of the valency of ions, and of the concentration of neutral salts on the viscosity of *suspensions* of finely powdered gelatin in water is similar to the influence of these three agencies on the viscosity of gelatin *solutions*; second, that the influence of electrolytes on the viscosity of the suspensions is due to the variation of the swelling (or relative volume) of the suspended particles; and third, that this latter fact explains why the Donnan equilibrium determines also the variation of viscosity of these suspensions. If it could be shown that a solution of gelatin contains also some (submicroscopic) particles of solid jelly (capable of swelling), we should understand at once why electrolytes influence the viscosity of gelatin solutions in a similar way as they influence the swelling, osmotic pressure, or the P.D. of these solutions. We have only indirect means of testing this occlusion theory of viscosity in the case of gelatin.

2. If these ideas are correct it would follow that if we melt a suspension of 0.5 gm. of powdered gelatin, in 100 cc. of water the viscosity of the 0.5 per cent solution of gelatin should be considerably lower than the viscosity of the suspension if measured at the same temperature, since in melting, part of the solid particles of the suspension should be transformed into individual molecules or ions or into particles too small to occlude water. In other words, as a consequence of the melting there should be a diminution of the relative volume occupied by the solid gelatin held originally in suspension.

This experiment was tried in the following way: 0.5 gm. of powdered gelatin was put into 100 cc. of water containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12.5, 15, and 20 cc. of 0.1 N HCl to bring the gelatin to different pH. The suspension was allowed to stand 1 hour at 20° to bring about the swelling of the particles and the viscosity of the suspension was measured in a straight viscometer at 20°C. The time of outflow of water through the viscometer at 20° was 48.5 seconds. The upper curve in Fig. 2 gives the ratio of viscosity of suspensions to that of water at 20°C. (When the viscosity is high, the values obtained are a little too great owing to a gravity effect which causes the solid particles to collect above the upper opening of the capillary tube during a part of the time of the experiment, thus increasing

temporarily the density of the suspension.) After the viscosity of a suspension was measured, the suspension was transformed into a solution by heating the suspension to 45° for 10 minutes; after that the solution was rapidly cooled to 20° and the viscosity of the gelatin solution was now measured with the same viscometer. The lower curve in Fig. 2 shows that the viscosity was now considerably diminished. The abscissæ are the pH of the gelatin solutions.

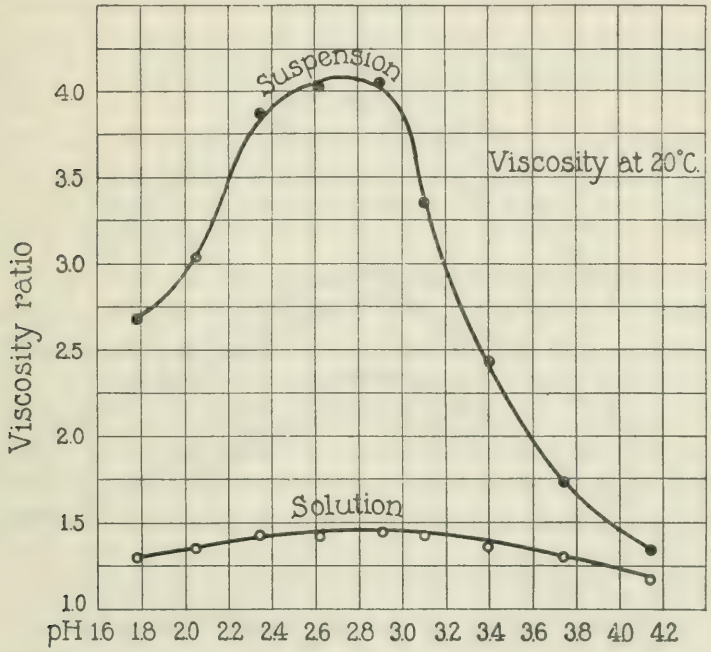

FIG. 2. Difference in the viscosity of a suspension of 0.5 gm. of powdered gelatin in 100 cc. and of the solution of the suspension in the same liquid; both viscosities were measured at 20°C.

Another test of the occlusion theory of viscosity is as follows. When we heat a 1 per cent solution of isoelectric gelatin rapidly to 45° and cool it rapidly to a lower temperature, *e.g.*, 20°C., the solution will ultimately set to a continuous gel but will steadily increase its viscosity before this stage is reached. It is natural to assume that the formation of a continuous jelly is preceded by the formation of smaller masses of jelly, which at first are submicroscopic and later

touch each other, forming a continous jelly. Hence the longer a solution of isoelectric gelatin stands at 20°C. the greater the number of submicroscopic solid pieces of jelly formed in the solution. The submicroscopic pieces of jelly surrounded by a true solution of isolated molecules of gelatin in water are compelled to regulate the amount of water they occlude by the Donnan equilibrium. Hence when we add some HCl to a 1 per cent solution of isoelectric gelatin after

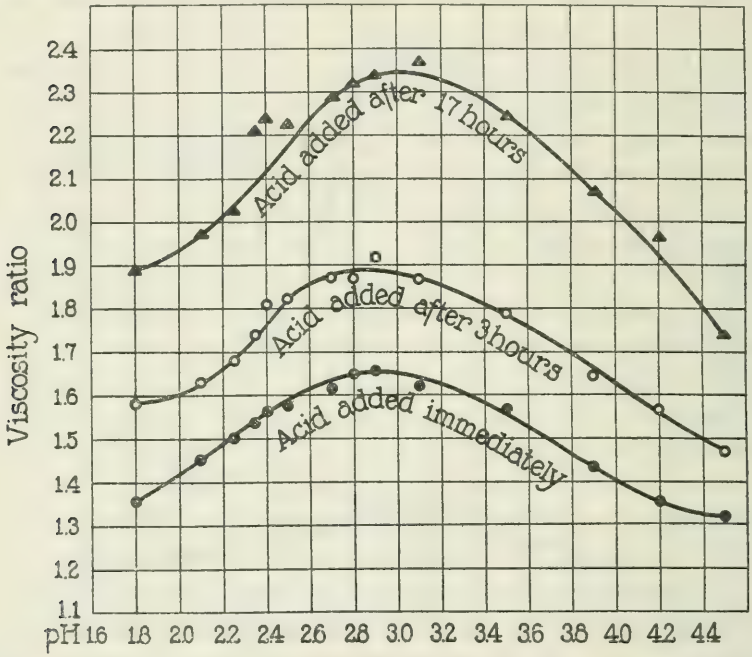

FIG. 3. Increase in viscosity when acid is added to solutions of isoelectric gelatin after they had been standing for 3 and 17 hours respectively.

the solution had been standing for some hours at 20° we should expect to find a higher viscosity than when we add the same amount of acid to the gelatin solution immediately after it had been rapidly heated to 45°C. and rapidly cooled to 20°C.

This experiment turns out as expected, as is shown in Fig. 3. When we bring newly prepared 0.5 per cent solutions of isoelectric gelatin to different pH by the addition of HCl we obtain a viscosity curve like the lowest in Fig. 3. When, however, we let the 0.5 per cent iso-

electric gelatin solution stand for 3 hours at 20°C. before adding the acid, we get a parallel curve but higher than the first one (middle curve, Fig. 3), for the reason that during the 3 hours an additional number of solid jelly particles capable of swelling has been formed. If we let the solution of isoelectric gelatin stand for 17 hours at 20°C., the curve (upper curve, Fig. 3), is still higher though practically parallel with the first curve except at the summit. It is probable that on standing the size of individual particles also increases and the greater the size the greater the viscosity, since the viscosity is chiefly but not exclusively a function of the relative volume of the particles.

These and similar experiments agree with the occlusion theory but not with the hydration theory; since there is no reason why the degree of hydration of gelatin should increase the longer isoelectric gelatin is kept at 20°C.

3. The fact that the viscosity of certain protein solutions is comparatively high, especially in the case of gelatin, has led to the suggestion that proteins might possess a different type of viscosity not present in solutions of crystalloids and that this second type of viscosity might be connected with a certain structure in the protein solution.

"Bearing in mind the possibility that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular and ionic motion. Thus a netlike structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance."[5]

[5] Robertson, T. B., The physical chemistry of proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 324–325.

This assumption of a second type of viscosity seems unnecessary since it is possible to account for the viscosities of protein solutions on the basis of Einstein's law when the relative volume occupied by the protein in solution is small, and on the basis of Arrhenius's formula when the volume exceeds the limits within which Einstein's formula holds. According to our view the former is true when the protein exists in the solution exclusively or almost exclusively in the form of isolated molecules or ions or particles too small to occlude water and this seems to be the case for solutions of crystalline egg albumin, at ordinary temperature, at a pH above 1.0 and when the concentration is not excessive.

The viscosity of such protein solutions is not only of a low order of magnitude but is not influenced by electrolytes in the way as is, *e.g.*, their osmotic pressure. Where we have viscosities of a higher order of magnitude, as in the case of gelatin solutions, we notice that the viscosity is influenced by electrolytes in the same way as is the osmotic pressure of these solutions. In the case of such proteins we assume that both the high order of viscosity as well as the characteristic influence of electrolytes on the viscosity are due to the same cause; namely, the existence in the gelatin solution of a certain number of submicroscopic particles of solid jelly occluding large masses of water, the exact amount of which is regulated by the Donnan equilibrium. The occlusion of large masses of water increases the relative volume occupied by the gelatin in solution so that Einstein's formula is no longer applicable. We are dealing, however, in both cases with the same type of viscosity which is primarily a function of the relative volume occupied by the protein particles in the solution.

Measurements of the influence of concentration on the viscosity of solutions of crystalline egg albumin of pH 5.1, *i.e.*, quite near the isoelectric point, were carried out at 15°C. and showed that the viscosity is under these conditions practically a linear function of the concentration (Fig. 4). If Einstein's formula $\frac{\eta}{\eta_0} = 1 + 2.5\ \varphi$ can account for the measurements, it should be possible to calculate the volume φ of the albumin in 100 cc. of solution from

$$\varphi = \left(\frac{\eta}{\eta} - 1\right)\frac{100}{2.5}$$

By dividing the weight of albumin in solution by its volume, we should obtain the density of albumin which should be according to the direct determination not far from 1.36 (Arrhenius).[4] Table II shows that if we calculate the density of solutions of albumin chloride on the basis of Einstein's formula we obtain values which only vary inside the limits of accuracy of the experiments from the density deter-

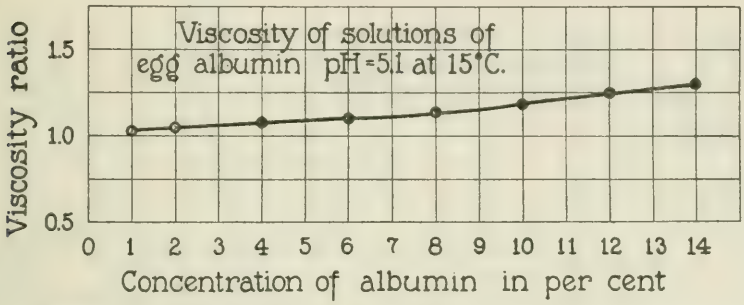
Viscosity of solutions of
egg albumin pH=5.1 at 15°C.
Viscosity ratio
1.5
1.0
0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Concentration of albumin in per cent

FIG. 4. Viscosity ratio of solutions of crystalline egg albumin near the isoelectric point. Inside the concentrations used, the viscosity ratio is nearly a linear function of the concentration.

TABLE II.

Concentration of crystalline egg albumin.	$\frac{\eta}{\eta_0} - 1$	Calculated volume of albumin.	Calculated density of albumin.
per cent		*cc.*	
14	0.290	11.6	1.20
12	0.240	9.6	1.25
10	0.185	7.4	1.35
8	0.132	5.3	1.51
6	0.100	4.0	1.50
4	0.074	2.96	1.36
2	0.042	1.7	1.17

mined directly. The time of outflow of water through the viscometer was 227 seconds at 15°C.

Entirely different results should be expected in the case of solutions of gelatin if the high viscosity is due to swollen particles of solid jelly contained in the solution and not due to the existence of a network, as some authors assume. When we plot the curve for the influence of concentration on the viscosity of *solutions of isoelectric gelatin* we get

curves like those of Fig. 5, which are no longer straight lines as in the case of crystalline egg albumin but curved lines. The curvature is the smaller the higher the temperature.

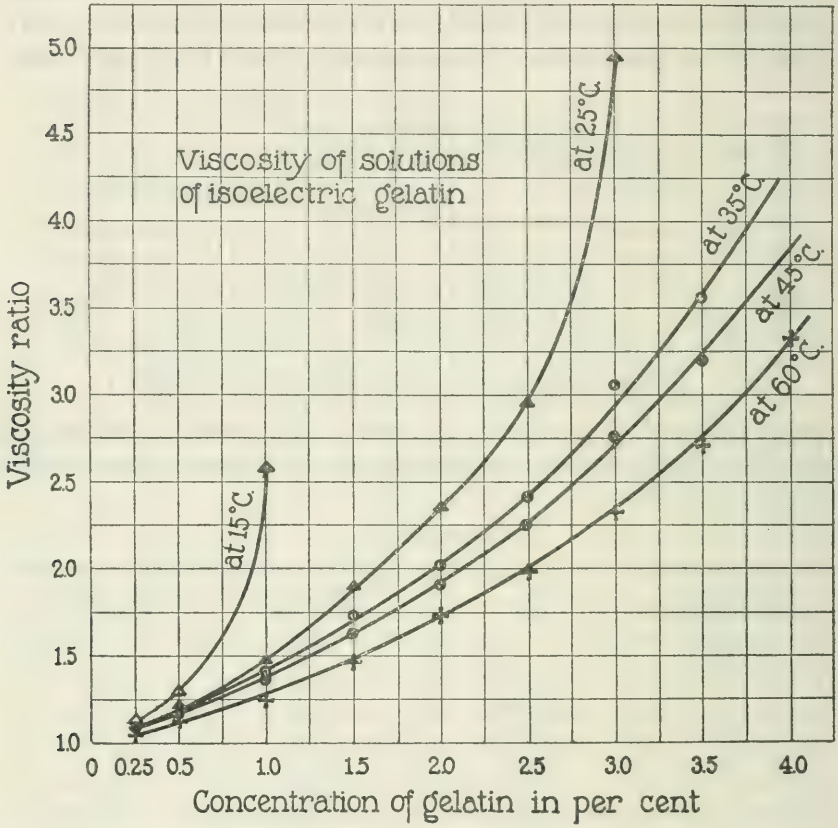

FIG. 5. Influence of concentration on the viscosity of solutions of isoelectric gelatin.

In these experiments the gelatin solution was first heated rapidly to 45° and then brought rapidly to the temperature indicated in the figure. The formula of Einstein can no longer be applied but the formula of Arrhenius gives fair agreement

$$\text{Log}\,\frac{\eta}{\eta} = \vartheta\varphi$$

where φ is the relative volume and ϑ is a constant. In other words, if Arrhenius's formula can account for the viscosity of these solutions the logarithm of the viscosity ratio when plotted over the concentration (Fig. 6), should give a straight line. The agreement of the value for 45° and 35° with this theory is satisfactory (considering the limits of accuracy of the measurements), (Fig. 6) the logarithms

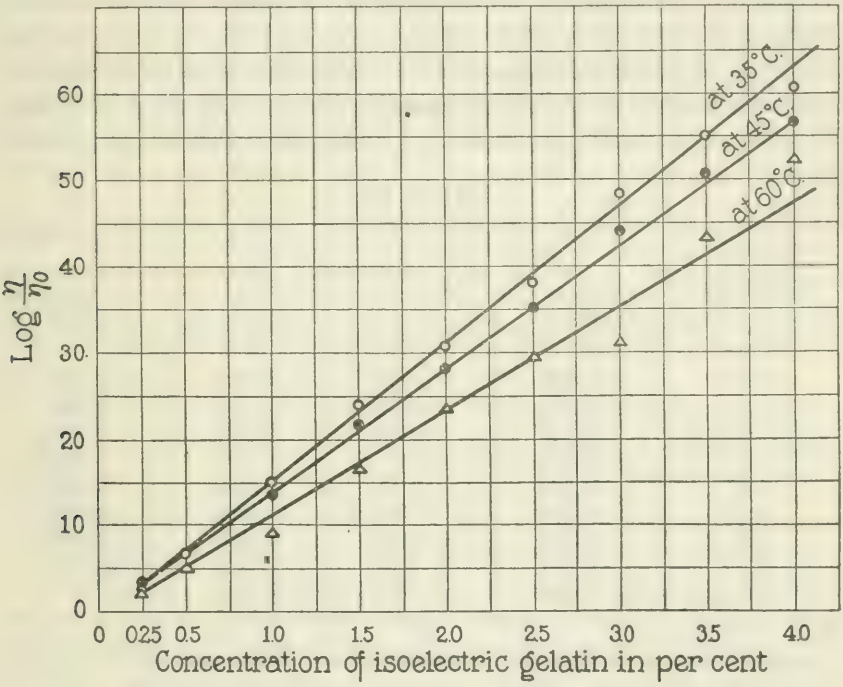

FIG. 6. Showing that the logarithms of the viscosity of solutions of isoelectric gelatin of different concentrations when plotted over the concentration as abscissæ are straight lines.

of the viscosity increasing practically in direct proportion with the concentration (*i.e.*, the relative volume) of the gelatin in the solution (Table III). At 60° the agreement is not quite so good but still recognizable. At 25°, however, it is satisfactory only at the lowest concentrations but at the higher concentrations the viscosity grows more rapidly than the concentration. The reason for this, however, is obvious since at this temperature the gelatin solution solidifies

so rapidly that the viscosity measurements were no longer possible for a concentration of 3.5 per cent gelatin solution, and for this reason the value of the viscosity of a 2 or 3 per cent solution is already too high on account of the mechanical hindrances of the flow of the solution through the viscometer owing to partial solidification.

From these experiments we may therefore draw the conclusion that since the viscosity measurements of gelatin solutions agree fairly well with Arrhenius's viscosity formula the variation of the viscosity of gelatin solutions must be due to variations in the relative volume occupied by the gelatin in solution; and since the gelatin used in these experiments was isoelectric, these variations cannot possibly

TABLE III.

Concentration of solution of isoelectric gelatin.	$\frac{\eta}{\eta_0}$			
	60°C.	45°C.	35°C.	25°C.
per cent				
0.25	0.0236	0.0306	0.0269	0.0374
0.5	0.0504	0.0682	0.0682	0.0792
1.0	0.0930	0.1350	0.1475	0.1685
1.5	0.1656	0.2135	0.2367	0.2765
2.0	0.2350	0.2796	0.3057	0.3701
2.5	0.2953	0.3512	0.3811	0.4691
3.0	0.3094	0.4409	0.4832	0.6941
3.5	0.4321	0.5051	0.5514	Solidifies
4.0	0.5214	0.5660	0.6043	

be ascribed to a hydration of gelatin ions. The only other way by which the isoelectric gelatin particles can increase their volume is through the occlusion of water by submicroscopic particles of solid jelly. Since the amount of water occluded by solid jelly is regulated by the Donnan equilibrium this will explain why the influence of electrolytes on the viscosity of a gelatin *solution* is similar to that of electrolytes on the viscosity of *suspensions* of powdered gelatin in water.

The connection between the tendency of gelatin solutions to set to a jelly and their high viscosity lies therefore in this, that the setting to a jelly is preceded by the formation of submicroscopic solid particles of gelatin capable of swelling and of occluding comparatively large

quantities of water whereby the relative volume occupied by the gelatin in the solution is enormously increased. That this interpretation is correct is proved by experiments on the viscosity of solutions of casein chloride which have no tendency to set to a jelly but which contain, side by side with isolated casein ions and molecules, small particles in suspension, occluding water and capable of swelling.

4. It has already been shown in a preceding paper that the pH influences the viscosity of casein solutions in a similar way as it influences the viscosity of gelatin solutions, and we have convinced ourselves that the addition of neutral salts has a similar depressing effect on the viscosity of casein chloride solutions as it has on the viscosity of solutions of gelatin chloride. Yet casein solutions have no tendency to set to a jelly (as far as the writer has been able to observe) and we cannot therefore attribute the influence of electrolytes on the viscosity of casein chloride solutions to a second type of viscosity. But in the case of casein chloride solutions we can prove directly that the influence of electrolytes on the viscosity has its source in the swelling of casein particles.

The material used in our experiments was a fine dry powder of nearly isoelectric casein prepared after Van Slyke and Baker.[6] Particles of equal size of grain (between mesh 100 and 120) were sifted out and 1 gm. of such powder was put into 100 cc. each of solutions of HCl of different concentration to bring the casein to varying pH.[7] A microscopic examination of the granules showed that they underwent a swelling which was a minimum at the isoelectric point, which increased with increasing hydrogen ion concentration until it reached a maximum and which then diminished again with a further increase in the hydrogen ion concentration. Hence the volume of the casein particles suspended in the HCl varied in a similar way with the pH as the volume of suspended particles of gelatin.

This swelling could also be observed when the suspension was put into 100 cc. graduates and the suspended particles were allowed to settle. The volume of the sediment was a minimum at the isoelectric

[6] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.
[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 547.

point increasing with increasing hydrogen ion concentration of the solution and finally decreasing again. But the curves for swelling and for volume of sediment were only parallel at the beginning, since the swelling (which occurred at once) was followed by some of the casein going into solution, or into suspension in the supernatant milky liquid. The longer the experiment lasted the smaller the volume of the sediment became and the larger the mass which went into the supernatant solution. This is expressed in Fig. 7. The upper curve represents the volume of the sediment after 1 hour. The

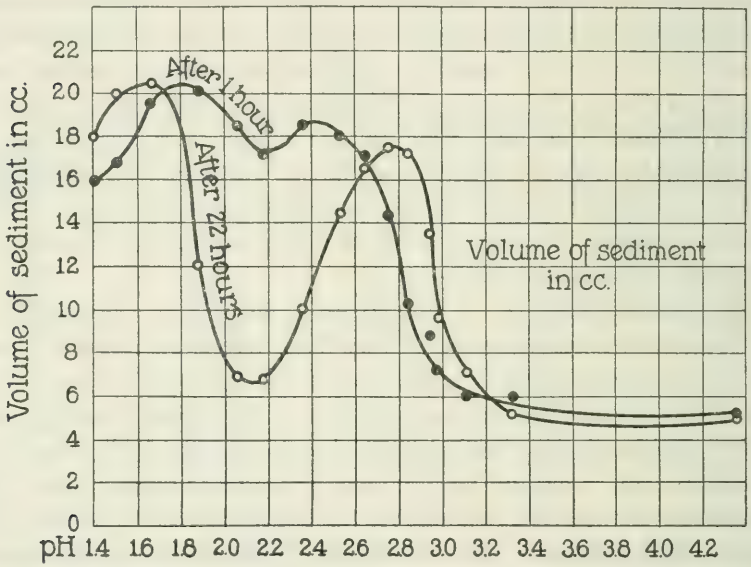

FIG. 7. Swelling and solution of casein chloride in 1 and 22 hours at 20°C.

suspension of 1 gm. of casein in 100 cc. HCl of different concentration had been kept for 1 hour at 20°, had been shaken repeatedly but not frequently and the suspension was then passed into 100 cc. graduates and allowed to settle at 20°C. After 2 hours the volume of the sediment was measured and the volumes are the ordinates of the curve marked "after 1 hour" in Fig. 7. A similar experiment was made in which the suspension of casein was kept for 22 hours at 20°C. and was allowed to settle during 6 hours also at 20°. The volumes are the ordinates of the second curve in Fig. 7 marked "after 22 hours." The abscissæ are the pH of the total solution and suspension.

The curve "after 1 hour" is clear, since it is chiefly the expression of the variation of the degree of swelling of the casein particles since not as much has gone into solution as after 22 hours. We notice that the volume occupied by the solid particles in the 1 hour curve is a minimum at the isoelectric point, that it rises steeply after pH 3.1, and it drops at 2.2, and that a second drop commences at pH 1.8. The two drops have a different cause. The drop at pH 1.8 is due to a diminution of the degree of swelling of the sediment, while the drop at 2.2 in the 1 hour curve is due to the fact that at 2.2 the solubility of casein chloride is a maximum. This conclusion is sup-

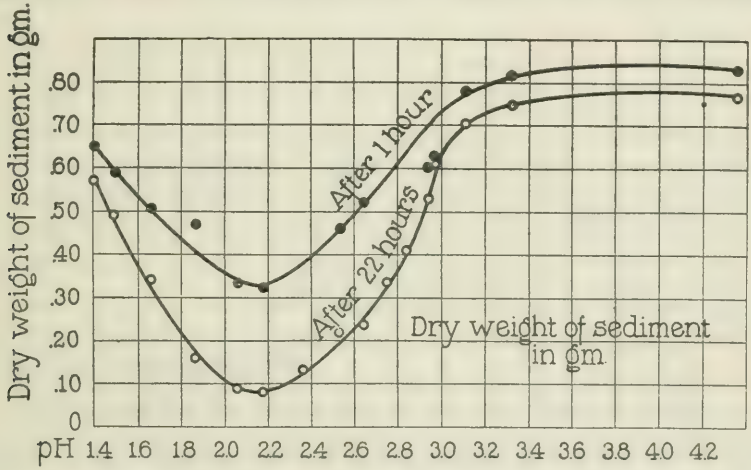

Fig. 8. Dry weight of sediment of casein chloride solutions after 1 and after 22 hours.

ported by the fact that the drop at 2.2 increases in time and is very considerable after 22 hours (Fig. 7), while otherwise the 1 hour and the 22 hour curves show only minor differences.

The proof that this interpretation in the volume curves of Fig. 7 is correct is furnished by Fig. 8 where the ordinates are the dry weights of the sediments, the volumes of which are given in Fig. 7. 1 gm. of powdered casein had when dried for 24 hours at between 90 and 100° a dry weight of 0.87 gm.

That part of the casein chloride which goes into the supernatant liquid (*i.e.*, which is not contained in the sediment) consists of two

constituents, namely, first, solid submicroscopic (and microscopic) particles in suspension which in due time would have settled, and second, isolated casein ions and molecules. The solid particles in the supernatant liquid (unless they are below the limit required to occlude water) undergo the same swelling under the influence of the Donnan equilibrium as the particles of the sediment. In addition, however, we have individual casein ions in solution (the molecules being probably insoluble since isoelectric casein is practically insoluble) but these ions cannot undergo any swelling and hence do not add materially to the volume and the viscosity. As a consequence the more solid particles of casein chloride are dissolved into isolated casein

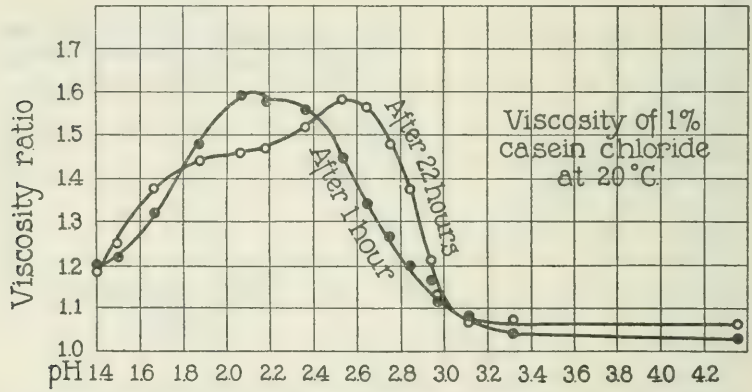

FIG. 9. Viscosity of 1 per cent casein chloride solutions after 1 and 22 hours at 20°C.

ions or particles too small to occlude water the more the relative volume occupied by the casein in the solution should be diminished and this should be accompanied by a diminution in viscosity. If our theory of the origin of the viscosity of the gelatin solutions is correct, it should be possible to prove that where the solubility of the casein chloride solution is a maximum the viscosity curve should show a drop.

The correctness of this inference is supported by the viscosity curves in Fig. 9 which represent the viscosity after 1 hour and after 22 hours. The experiments are the same as those referred to in Figs. 7 and 8. The viscosity of the total suspension and solution

was measured in a straight viscometer with a time of outflow for water of 48.4 seconds at 20°C. The curve for the viscosities after 1 hour is the expression chiefly of the swelling, since casein chloride goes only slowly into solution at 20°C. The curve is almost continuous and has its maximum in the region between pH 2.1 and 2.4, where also the swelling is a maximum. There is, however, a slight depression at pH 2.2 where the solubility of the casein is a maximum.

The curve for the viscosities after 22 hours shows, however, a distinct saddle at pH 2.2 where the solubility of casein chloride is a maximum. This agrees with the assumption that the high viscosity is due to swollen particles of casein, a certain quantity of which had been dissolved at or near pH of 2.2. This solution of the particles capable of swelling beneath that size where they no longer can occlude water must diminish the relative volume of the casein and cause a diminution of the viscosity. Below a pH of 1.8 where the solubility of the casein is considerably diminished, the 1 hour and the 22 hours viscosity curves (Fig. 9) no longer differ materially. As a consequence of the saddle the maximum of the viscosity curve after 22 hours now lies at pH 2.6.

Since the point at issue, namely the diminution of the viscosity when solid submicroscopic particles, capable of swelling, are dissolved into particles so small that they no longer can occlude water is so fundamental for the theories of viscosity and of colloidal behavior in general that it seemed necessary to look for a more striking proof than that given in the experiment quoted. For this purpose measurements were made on 1 per cent casein chloride solutions prepared from very finely powdered casein particles sifted through a 200 mesh. In order to get a more rapid dispersion of the particles the experiment was carried out at 40°C. The time of outflow of water through the viscometer at 40° was 35.5 seconds. Fig. 10 gives the results. The viscosity measurements were made at four different times; namely, first, immediately after the powdered casein was put into the HCl; then after 1, 3, and 6 hours. During this time the casein chloride solutions were kept at 40°C. The viscosity curve taken immediately after the suspensions were prepared is continuous and is the expression of the swelling which occurred in the few minutes which elapsed in the preparation of the suspensions and during

which the casein was at 40°C. The maximum swelling occurred at about pH 2.3. At this time the amount of casein dissolved into separate casein ions was negligible. The curve resembles the 1 hour curve in Fig. 9 except that the depression due to the solution of some casein chloride in Fig. 9 is lacking in Fig. 10. After $1\frac{1}{2}$ hours the second measurements of viscosity were taken, and the reader will notice from Fig. 10 that the viscosity had dropped considerably in the neighborhood of pH. 2.2, where the solubility of casein chloride is the greatest, and the maximum depression is at pH 2.1 where also the solubility is a maximum. With a further lowering of the pH the viscosity rises again. The maximal viscosity in the $1\frac{1}{2}$ hours

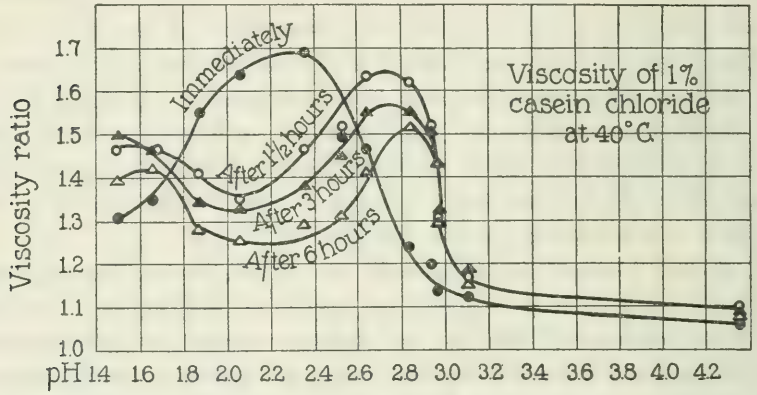

FIG. 10. Diminution of viscosity through solution of solid particles of casein chloride.

series is now at pH of about 2.7 or 2.8 where it was also in 22 hours series in Fig. 9. The later viscosity measurements, after 3 and 6 hours (Fig. 10), confirm these conclusions.

5. These experiments leave little doubt that the high viscosity of protein solutions is due to the existence of particles occluding large quantities of water the amount of which is regulated by the Donnan equilibrium while the isolated ions of proteins in solution or the particles too small to occlude water have no share in the causation of high viscosities.

The quantities of water which can be occluded in a solid jelly of gelatin are enormous. If we assume the molecular weight of gelatin

to be of the order of magnitude of about 12,000 a solid gel of 1 per cent originally isœlectric gelatin contains over 60,000 molecules of water to 1 molecule of gelatin. It is out of the question that such masses of water could be held by the secondary valency forces of the gelatin and water molecules. Swelling casein particles occlude much less water and for this reason the viscosity of casein chloride solutions never becomes as high as that of gelatin solutions, containing equal masses of protein per 100 cc. of solution.

It is well known (and we have made use of the fact earlier in this paper) that the viscosity of a gelatin solution that had been heated to 45°C. and is allowed to stand at 20° increases steadily even if the concentration is not high enough to permit the setting of the solution to a jelly. This increase in viscosity on standing seems to be caused by the gradual increase in the number of solid particles of jelly due to the collision of gelatin molecules or ions, these solid particles occluding water and thus increasing the apparent volume occupied by the gelatin in the solution. The gradual formation of solid particles of jelly from isolated molecules is most rapid at the isœlectric point and is retarded by the addition of acid; while the addition of acid increases the swelling of those particles of jelly which already exist in the solution. It is well to keep in mind that the addition of acid to a solution of isœlectric gelatin has thus two opposite effects on the viscosity of gelatin solutions.

According to Zsigmondy[8] Smoluchowski has explained (in a paper which has not been accessible to the writer) the increase in the viscosity through coagulation of a solution of aluminium oxide by the assumption of an occlusion of liquid between the particles. Smoluchowski calculates from the increase of viscosity during coagulation of aluminium oxide that the coagulating particles occupy a volume 400 to 500 times as great as that occupied by the dry material itself. This apparent increase of volume he explained through the aggregation of needleshaped particles. It would be of interest to test whether or not the volume of these particles and the viscosity of the suspension of aluminium oxide is also controlled by the Donnan equilibrium.

We are therefore led to a conception of the nature of protein solutions which is somewhat different from that current in the literature of

[8] Zsigmondy, R., *Kolloidchemie*, Leipsig, 2nd ed., 1918, 98.

colloid chemistry, which assumes that proteins form no true solutions but suspensions or emulsions. According to our view protein salts form, as a rule, true solutions consisting of isolated protein ions and molecules, which, however, may contain in addition solid fragments occluding comparatively large quantities of water. These latter particles of solid material are responsible for both the comparatively high viscosity of solutions of certain proteins, and for the influence of electrolytes on viscosity.

6. It is of interest to see whether or not Arrhenius's formula can account for the influence of electrolytes on the viscosity of casein suspensions. If this were the case, the curves representing *Log* $\frac{\eta}{\eta_0}$ should run parallel to curves representing the relative volume occupied by the casein in the solution. We get the values of Log $\frac{\eta}{\eta}$, from our observations of the relative viscosity which give us $\frac{\eta}{\eta_0}$, and we can calculate the volume from the measured volume of the sediment plus the calculated volume of the casein in the supernatant liquid. The latter value we obtain by deducting the dry weight of the sediment from the (known) dry weight of the whole mass of casein put into the water (1 gm. powdered casein, dry weight = 0.87 gm.), and assuming that the casein in the supernatant liquid consists exclusively of suspended particles. This is approximately correct for a 1 hour experiment at 20°. The ordinates in Fig. 11 represent the values for volume thus corrected and the values for log $\frac{\eta}{\eta_0}$ while the abscissæ are the pH of the suspensions. The two curves are almost parallel.

It should be stated that these corrected volumes of casein include a certain amount of water between the granules. We are, however, in this case not concerned with the absolute but the relative volume occupied by the casein.

When we add NaCl in different concentrations to a casein chloride solution we notice that the viscosity is diminished as it is in the case of solutions of gelatin chloride. R. F. Loeb found by microscopic observation that this diminution of viscosity was accompanied by a diminution in the degree of swelling of the individual particles of casein which ran parallel to the depression of the viscosity.

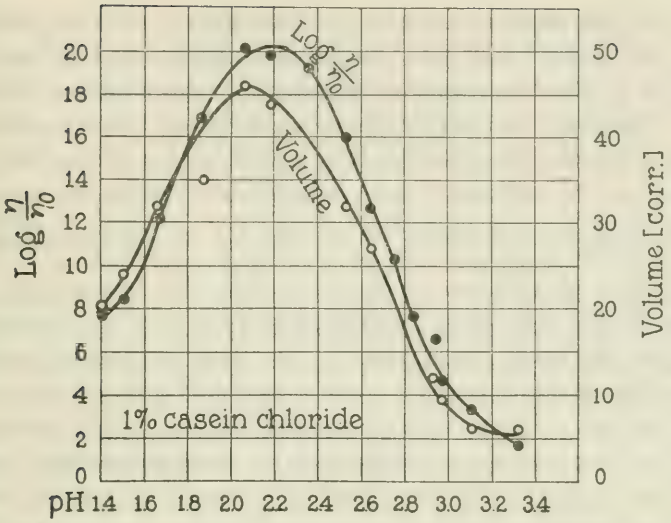

FIG. 11. Similarity of curve for log $\frac{\eta}{\eta_0}$ and of relative volume of casein chloride solutions.

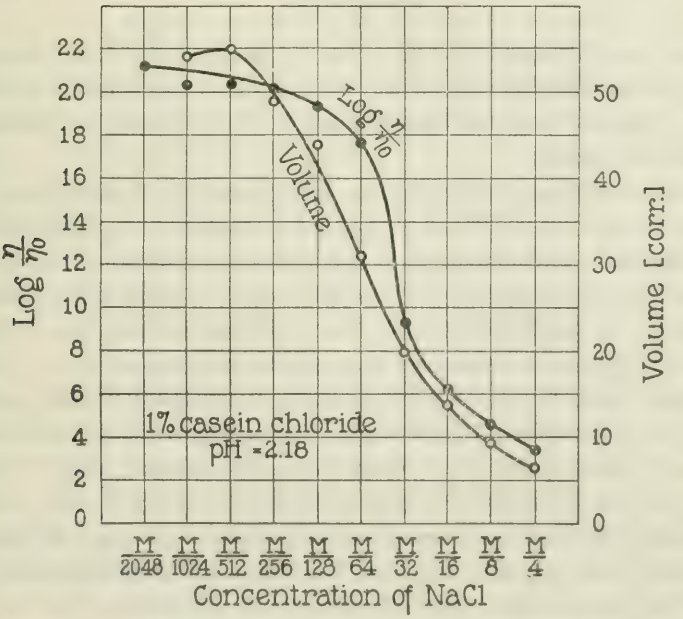

FIG. 12. Similarity of curve for log $\frac{\eta}{\eta_0}$ and of relative volume of casein chloride solutions.

1 gm. of powdered casein was put into 100 cc. of H_2O containing 12.5 cc. of 0.1 HCl and NaCl in concentrations varying from 0 to M_4. The mixture was shaken occasionally and kept for 16 hours at 20°. Then the viscosity, volume of sediment (after settling for 24 hours), dry weight of sediment (after deduction of the free NaCl contained in the sediment) were determined. When the corrected volume and the values for $\log \frac{\eta}{\eta_0}$ are plotted as ordinates over the concentration of abscissæ it is found that the two curves agree fairly well (Fig. 12) except where no or little salt was added and where therefore some casein particles had been completely dissolved. In this solution the calculated volume was too high and our curves express the fact. From these experiments we may conclude that the influence of electrolytes on the viscosity of casein solutions or suspensions is due to the swelling of particles of casein suspended in the solution of casein and that the volume of these particles is regulated by the Donnan equilibrium.

SUMMARY.

1. The proof is completed that the influence of electrolytes on the viscosity of suspensions of powdered particles of gelatin in water is similar to the influence of electrolytes on the viscosity of solutions of gelatin in water.

2. It has been suggested that the high viscosity of proteins is due to the existence of a different type of viscosity from that existing in crystalloids. It is shown that such an assumption is unnecessary and that the high viscosity of solutions of isoelectric gelatin can be accounted for quantitatively on the assumption that the relative volume of the gelatin in solution is comparatively high.

3. Since isoelectric gelatin is not ionized, the large volume cannot be due to a hydration of gelatin ions. It is suggested that this high volume of gelatin solutions is caused by the existence in the gelatin solution of submicroscopic pieces of solid gelatin occluding water, the relative quantity of which is regulated by the Donnan equilibrium. This would also explain why the influence of electrolytes on the viscosity of gelatin solutions is similar to the influence of electrolytes on the viscosity of suspensions of particles of gelatin.

4. This idea is supported by experiments on solutions and suspensions of casein chloride in which it is shown that their viscosity is chiefly due to the swelling of solid particles of casein, occluding quantities of water regulated by the Donnan equilibrium; and that the breaking up of these solid particles into smaller particles, no longer capable of swelling, diminishes the viscosity.

5. This leads to the idea that proteins form true solutions in water which in certain cases, however, contain, side by side with isolated ions and molecules, submicroscopic solid particles capable of occluding water whereby the relative volume and the viscosity of the solution is considerably increased. This accounts not only for the high order of magnitude of the viscosity of such protein solutions but also for the fact that the viscosity is influenced by electrolytes in a similar way as is the swelling of protein particles.

6. We therefore reach the conclusion that there are two sources for the viscosity of protein solutions; one due to the isolated protein ions and molecules, and the other to the submicroscopic solid particles contained in the solution. The viscosity due to the isolated molecules and ions of proteins we will call the general viscosity since it is of a similar low order of magnitude as that of crystalloids in solution; while the high viscosity due to the submicroscopic solid protein particles capable of occluding water and of swelling we will call the special viscosity of protein solutions. Under ordinary conditions of hydrogen ion concentration and temperature (and in not too high a concentration of the protein in solution) the general viscosity due to isolated ions and molecules prevails in solutions of crystalline egg albumin and in solutions of metal caseinates (where the metal is monovalent) while under the same conditions the second type of viscosity prevails in solutions of gelatin and in solutions of acid-salts of casein; and also in solutions of crystalline egg albumin at a pH below 1.0 and at higher temperatures. The special viscosity is higher in solutions of gelatin than of casein salts for the probable reason that the amount of water occluded by the submicroscopic solid gel particles in a gelatin solution is, as a rule, considerably higher than that occluded by the corresponding particles of casein.

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THE RECIPROCAL RELATION BETWEEN THE OSMOTIC PRESSURE AND THE VISCOSITY OF GELATIN SOLUTIONS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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1. If we put a suspension of 1 gm. of finely powdered gelatin in 100 cc. of HCl, so that the gelatin has a pH of 3.4, into a collodion bag and dip the latter into a pure solution of HCl (without gelatin) of pH 3.0, the liquid will practically not rise in the manometer connected with the liquid in the collodion bag (except to that slight extent which may be caused by some of the solid gelatin going into solution); in other words, we get no osmotic pressure. The reason is as follows: in this experiment the Donnan equilibrium is established between each individual granule of solid gelatin and the solution of HCl inside the bag and since the latter diffuses freely through the membrane there is in this case no difference of pH inside and outside the collodion membrane, and hence no difference of osmotic pressure on the opposite sides of the membrane (unless some of the solid gelatin goes into solution).

If we make this experiment, however, with a 1 per cent *solution* of gelatin chloride of a pH of 3.4 inside the collodion bag, while on the other side of the membrane we have a HCl solution of pH 3.0 without gelatin, we notice a rapid rise of the column of water in the manometer tube inserted into the collodion bag and the osmotic pressure of the 1 per cent solution of gelatin will be about 450 mm. H_2O or a little more when equilibrium is established.[1] This proves that the osmotic pressure of a gelatin solution cannot be due to (submicroscopic) solid particles of a jelly, but must be due to particles causing a Donnan equilibrium *across the collodion membrane.* Such particles can only be the individual gelatin ions, since even the smallest

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

particle of gel must cause a Donnan equilibrium to be established between the solid particles and the solution inside the bag.

If we assume that certain protein solutions contain two different constituents, namely, first, isolated ions and molecules, and second, isolated submicroscopic particles capable of occluding water, these two constituents of such solutions must play a different rôle in osmotic pressure and in viscosity. The characteristic influence of electrolytes on the osmotic pressure of protein solutions must be due to the isolated protein ions since these alone can bring about the Donnan equilibrium across the membrane; while the similar influence of electrolytes on the viscosity of protein solutions must be due to the solid submicroscopic particles since these alone are capable of swelling and of giving rise to a Donnan equilibrium between each individual particle and solution.[2] If this inference is correct, it should be possible to demonstrate the existence of a reciprocal relation between the viscosity and the osmotic pressure of those protein solutions which contain isolated protein ions as well as solid protein particles capable of occluding water; *e.g.*, gelatin or casein solutions. It is the purpose of this paper to show that this reciprocal relation between the osmotic pressure and viscosity of such protein solutions actually exists.

Fig. 1 shows that the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin diminishes the more, the more we replace the dissolved gelatin by small granules of powdered gelatin. The ordinates of the upper curve represent the values of the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin at different pH, the pH serving as abscissæ of the curves. The acid used was HCl, and the curve is the usual one given repeatedly in the writer's preceding publications. At the beginning of the experiment the gelatin solution was rapidly heated to a temperature of 45°C. and rapidly cooled to 20°C. and then kept at that temperature throughout the entire experiment. The pH is that of the gelatin solution at the end of the experiment.

The middle curve represents an experiment in which 0.5 gm. of the isoelectric gelatin in solution was replaced by 0.5 gm. of isoelectric powdered gelatin. The latter did not contribute to the osmotic

[2] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 73.

pressure, the observed osmotic pressure being due to the isolated ions of the 0.5. per cent gelatin in solution which determine the Donnan effect, and in addition, to the gas pressure of the isolated gelatin ions and the isolated gelatin molecules. Theoretically, of course, the

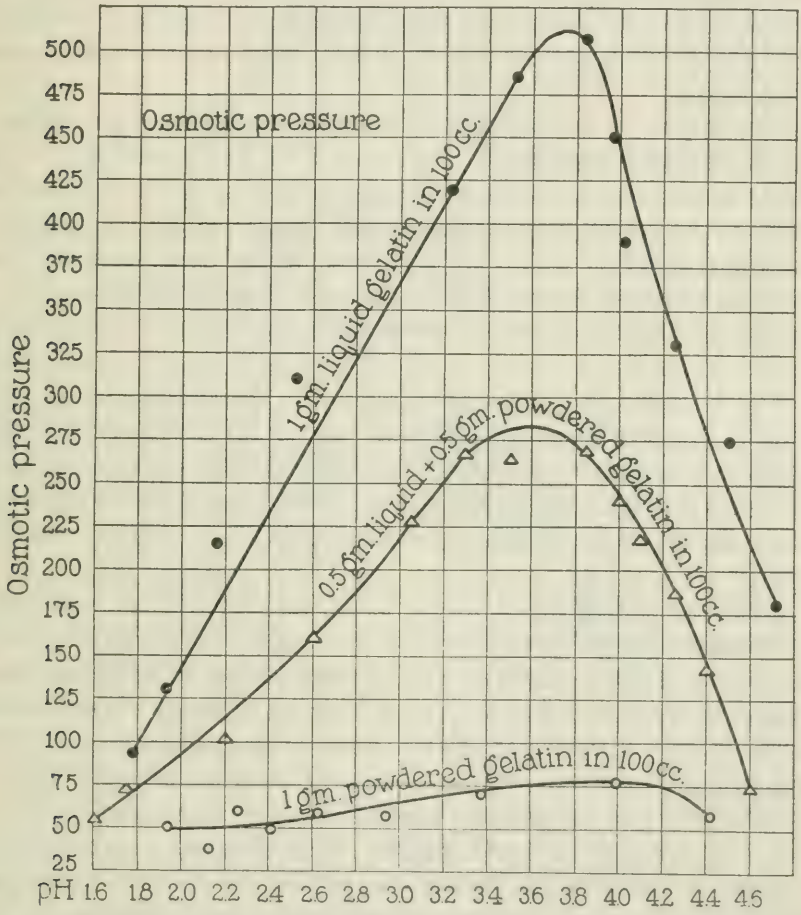

FIG. 1. A suspension of 1 gm. of a fine powder of gelatin in 100 cc. of water has practically no osmotic pressure (lowest curve), while a solution of 1 gm. of the same gelatin has a maximal osmotic pressure of over 500 mm. (uppermost curve). A mixture of 0.5 gm. of powdered and 0.5 gm. of liquid gelatin in 100 cc. water has practically the osmotic pressure of the 0.5 per cent liquid gelatin in 100 cc. of water (middle curve).

coarse particles of gelatin also participate in the osmotic pressure but this effect is negligible on account of the small number of such particles. (The gelatin particles used were of grain size slightly above one sixtieth of an inch diameter.) At the beginning of the experiment the 0.5 per cent solution of gelatin was rapidly heated to 45°C. and rapidly cooled to 20°C., and then the powdered gelatin was added. The pH is that of the 0.5 per cent gelatin solution at the end of the experiment.

The lowest curve represents the osmotic pressure of 1 gm. of powdered isoelectric particles in 100 cc. of HCl of different pH. The slight osmotic pressure observed is probably due to the fact that a little of the gelatin went gradually into solution. This apparently happened to a less extent in a repetition of this experiment and the osmotic pressures observed were still lower than in the lowest curve in Fig. 1. All these osmotic pressure experiments were made in a thermostat at 20°C.

The viscosity is affected in exactly the opposite way from the osmotic pressure if part of the dissolved gelatin is replaced by solid particles of gelatin. The more dissolved gelatin is replaced by solid particles of gelatin the higher the viscosity, a result to be expected from the experiments and conclusions published in the preceding paper.[2]

Solutions of 0.5, 0.625, 0.750, 0.875, and 1.0 gm. of isoelectric gelatin were heated quickly to 45°C. and cooled quickly to 20°C. and so much powdered gelatin of pH 7.0 was added as to bring the total gelatin in 100 cc. to 1 gm.; *i.e.*, to a 0.5 per cent solution of gelatin was added 0.5 gm. of powdered gelatin (between mesh sizes 100 and 120), and to a 0.875 per cent solution of liquid gelatin was added 0.125 gm. of powdered gelatin, while no powdered gelatin was added to the 1 per cent solution of liquid gelatin. The different mixtures were brought to different pH through the addition of different quantities of HCl and the solutions were allowed to stand for 1 hour before the viscosities were measured in order to give the powdered gelatin a chance to swell. The results of the measurements are represented in Fig. 2. The reader will see that within the range of the pH between 3.6 and 1.4 the viscosity is the greater, the more liquid gelatin is replaced by powdered gelatin. This supports the idea that the

influence of electrolytes on the viscosity of gelatin solutions is due to the influence of the electrolytes on the swelling of solid submicroscopic particles of gel in the solution.

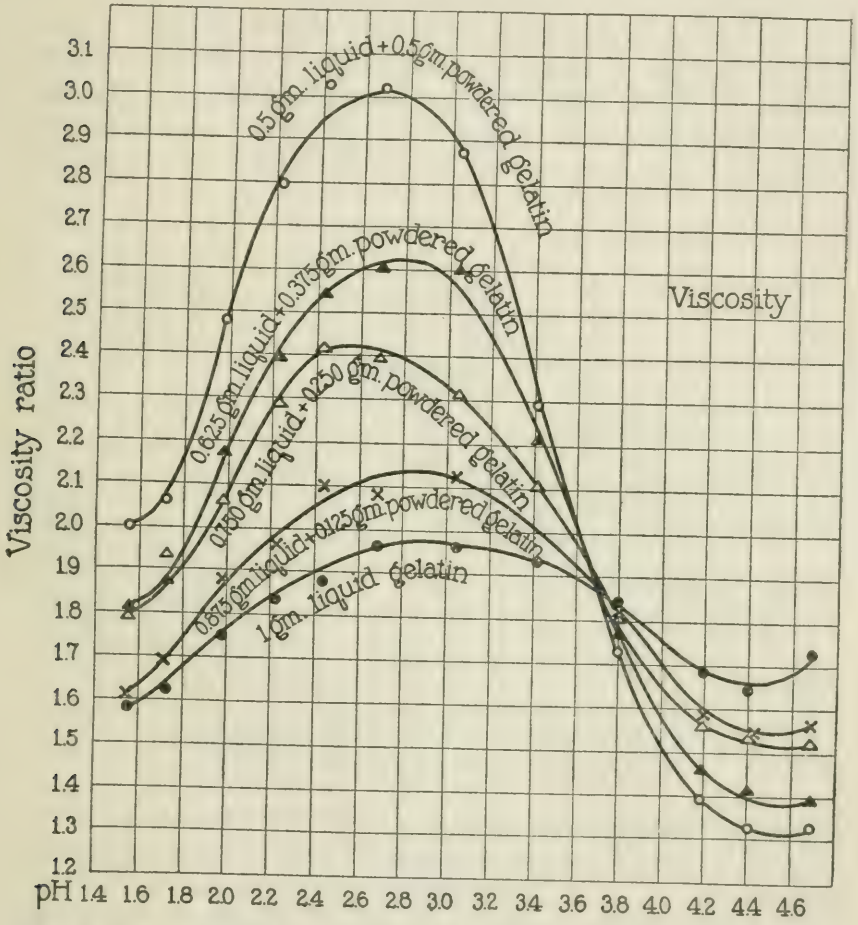
Viscosity ratio
pH
Viscosity
0.5 gm. liquid + 0.5 gm. powdered gelatin
0.625 gm. liquid + 0.375 gm. powdered gelatin
0.750 gm. liquid + 0.250 gm. powdered gelatin
0.875 gm. liquid + 0.125 gm. powdered gelatin
1 gm. liquid gelatin

FIG. 2. The influence of replacing liquid by powdered gelatin on viscosity is exactly the reverse as on osmotic pressure. The more the powdered particles replace the liquid gelatin the higher the specific viscosity.

The nature of the curves in Fig. 2 between pH 4.6 and 3.8 requires an explanation. The curves are here the lower the more liquid gelatin is replaced by solid gelatin. This is due to the fact that it was neces-

sary to let the suspensions stand for at least 1 hour to allow the particles of powdered gelatin to swell before the viscosity measurements were made. During this time the liquid gelatin at, or near, the isoelectric point increases rapidly in viscosity while this increase in viscosity is suppressed where the hydrogen ion concentration is higher. This is proved by Fig. 3 which gives the viscosity of the supernatant solutions of gelatin (without the suspended particles) which had been standing for 1 hour. Inside the range of pH 4.4 and 4.6 the viscosity had risen more rapidly on standing than at the lower pH;

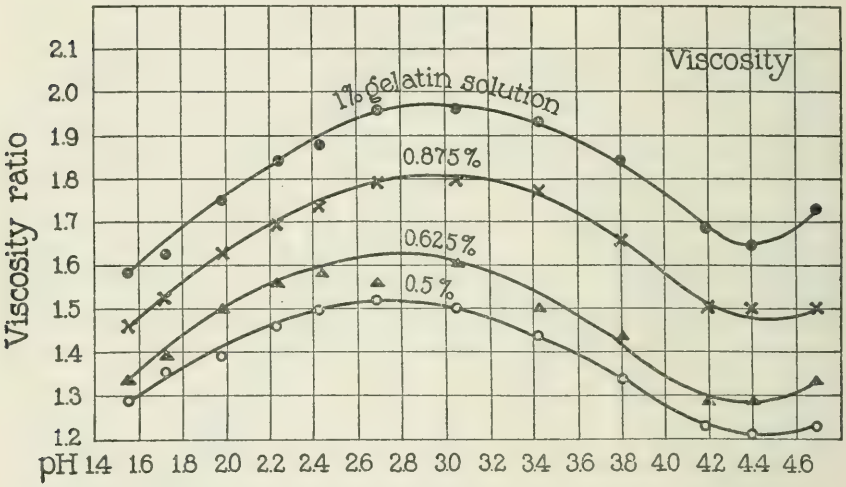

FIG. 3. Increase of viscosity of gelatin solution at the isoelectric point on standing due to the formation of submicroscopic particles of gel.

which means that at, or near, the isoelectric point new submicroscopic particles of solid jelly are constantly formed from the molecules while this process is the slower the higher the hydrogen ion concentration. While thus the addition of acid to a solution of isoelectric gelatin retards the rate of formation of new submicroscopic particles of jelly, it increases the swelling of those already present when the acid is added. On the other hand, powdered particles of isoelectric gelatin in water of pH 4.7 do not increase their volume on standing.

The fact that the addition of acid to a solution of isoelectric gelatin inhibits or retards the formation of new solid particles on standing

is strikingly illustrated by Fig. 4. Gelatin chloride solutions all containing 1 gm. of originally isoelectric gelatin were brought to various pH by adding HCl and were at the beginning of the experiment all heated rapidly to 45° and cooled at once to 20°C. Then the viscosity was measured immediately at 20°. The result is given in the lowest curve in Fig. 4. The solutions were kept at 20° for 1 hour and the viscosity measurements were repeated. The middle curve in Fig. 4

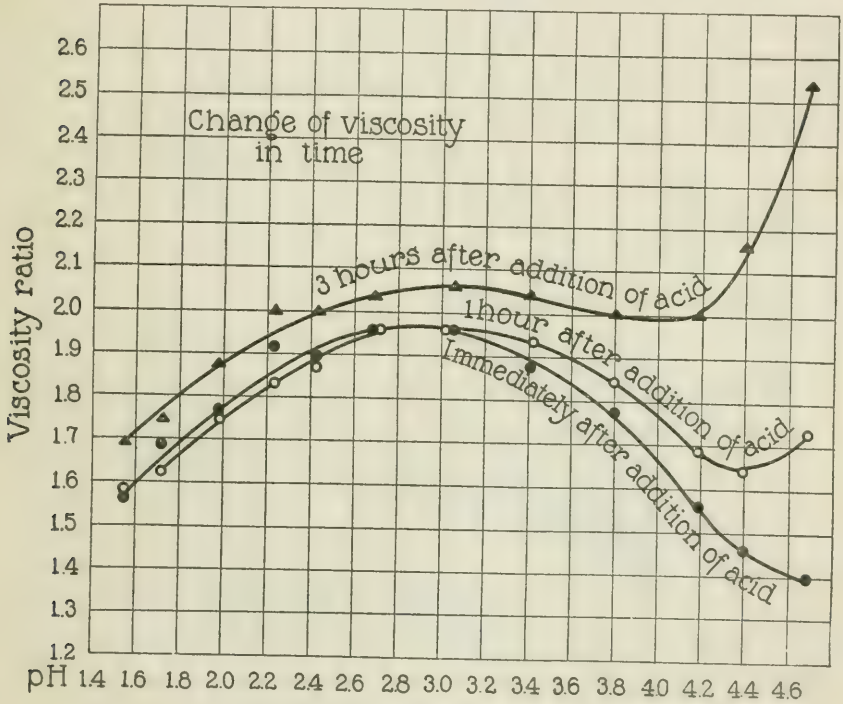

FIG. 4. See legend of Fig. 3.

shows that at this time the viscosity of the isoelectric solution had increased considerably, that of the solutions of lower pH down to 3.0 had increased the less, the lower the pH. Below pH 3.0 no increase in the viscosity had occurred. 2 hours later another viscosity measurement was made; the results are represented in the upper curve of Fig. 4. At the isoelectric point the viscosity had increased enormously, but less and less at lower pH. During all the time and also

during the viscosity measurements the temperature of the solutions was 20°C.

A more exact demonstration of the inhibiting influence of acid on the increase of viscosity of gelatin solutions on standing is given in

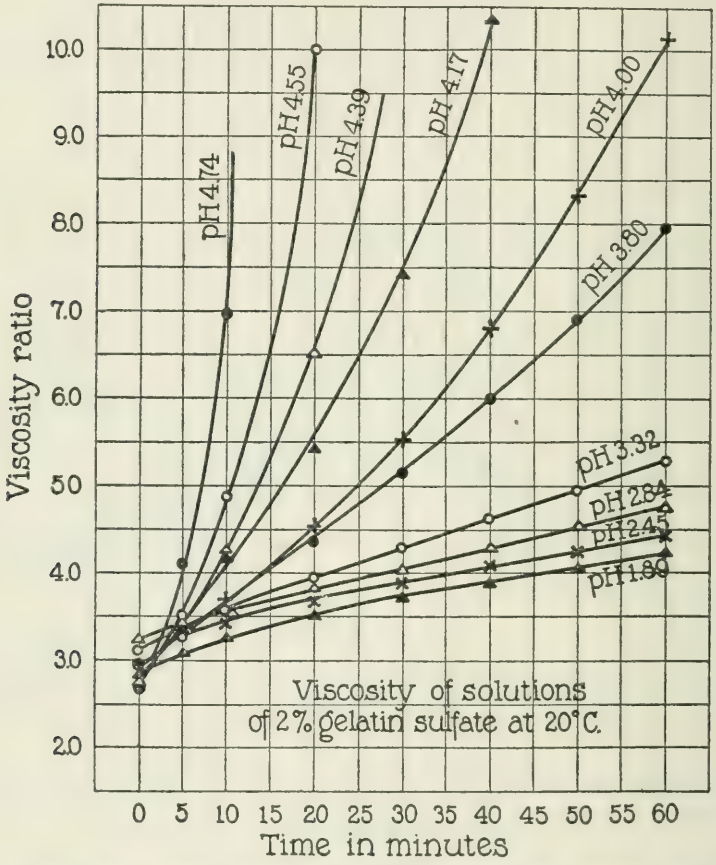

FIG. 5. Increase of viscosity of gelatin sulfate solution of different pH on standing. The increase is most rapid at the isoelectric point, thus proving that the acid retards or prevents the formation of submicroscopic solid particles of jelly on standing.

Fig. 5. Gelatin sulfate solutions containing 2 gm. of originally isoelectric gelatin in 100 cc. were prepared and heated rapidly to 45°C. and cooled rapidly to 20°C., and then kept constantly at 20°C.

Viscosity measurements were made every 5 or 10 minutes. Fig. 5 gives the result. The lower the pH the less the viscosity increases on standing.

There exist, therefore two different and to some extent antagonistic effects of acid on the viscosity of gelatin solution. One effect is due to the swelling of the submicroscopic particles of solid gelatin already present in the solution at the time the acid is added. This effect is regulated by the Donnan equilibrium and increases when (not too much) acid is added to the isoelectric gelatin solution. The second effect consists in retarding the formation of solid particles of jelly on standing. This inhibitory effect of the acid seems to increase steadily with the hydrogen ion concentration of the solution without going through a maximum at a pH above 1.8.

If we now return to the discussion of the curves in Fig. 2 we may say that the results in that part of the curves which belongs to the abscissæ of pH above 3.8 is the expression of the fact that that part of the viscosity which is due to the gelatin in solution had undergone an increase during the hour the solution had been standing at 20° after having been heated to 45°C.; and that the increase caused in the viscosity of the liquid gelatin was a maximum at the isoelectric point, being almost zero at a pH below 3.4; while the addition of acid had the opposite effect on the solid granules of gelatin, since their volume was increased according to the rules of the Donnan equilibrium.

It is necessary that we convince ourselves that a Donnan equilibrium exists when particles of solid gelatin are suspended in a solution of gelatin. That this is actually true was shown in the following way. 0.5 gm. of powdered gelatin was added to 100 cc. of a 0.5 per cent gelatin chloride solution of different pH. The different beakers containing these mixtures were kept for $3\frac{1}{2}$ hours at 20°C. The mass was then filtered through cotton wool and the pH of the filtrate (0.5 per cent gelatin solution) and of the solid gelatin granules were determined, that of the latter after they had been melted. It was found that the pH of the gelatin granules was higher than that of the solution and that the difference followed the Donnan equilibrium equation (Table I), though the result was slightly irregular owing to the fact that it is impossible to free the suspended particles of gelatin completely from the supernatant liquid. When we separate

TABLE I.

pH of gelatin in suspension....	5.12	4.60	4.49	4.18	4.07	3.73	3.45	2.93	2.68	2.34	2.09	1.86	1.77	1.53
pH of gelatin in solution.....	4.98	4.35	4.12	3.91	3.69	3.50	3.14	2.78	2.50	2.28	1.97	1.86	1.72	1.57

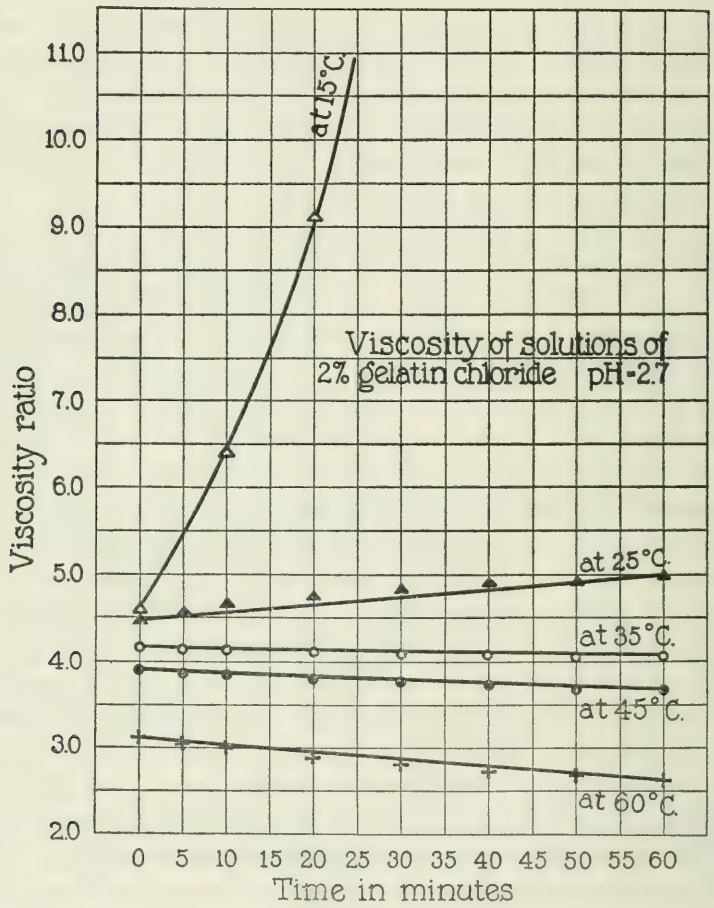

FIG. 6. Influence of temperature on the variation of viscosity of gelatin solutions on standing. Below 35°C. the viscosity of a 2 per cent gelatin chloride solution of pH 2.7 no longer increases but diminishes on standing.

a gelatin solution from water by a collodion membrane we get two equilibria, one across the membrane caused by the protein ions on one side of the membrane; and a second one inside the protein solution caused by the solid particles of jelly.

2. These experiments in which liquid gelatin was replaced by solid particles of gelatin support the idea of a reciprocal relation between the osmotic pressure and the viscosity of gelatin *solutions*. It is, however, possible to give a more direct proof. It was noticed that the viscosity of solutions of gelatin chloride does not always increase on standing but that it diminishes when the temperature exceeds a certain limit. This is shown for a 2 per cent solution of gelatin chloride of pH 2.7 in Fig. 6. The viscosity of such a solution increases on standing very rapidly at 15°C., much less rapidly at 25°C., but diminishes when kept at a temperature above 35°C., and the more rapidly the higher the temperature. This we assume to be due to the fact that at a temperature above 35° the rate of melting of submicroscopic particles of solid jelly exceeds the rate of their formation from isolated ions or molecules.

Several liters of a 0.55 per cent solution of isoelectric gelatin were kept at about 10°C. for 48 hours and at 20° for the next 24 hours. Then the stock solution was divided into two parts. The one part was divided into parts of 90 cc. each, and each part was brought to different pH by adding 10 cc. containing different quantities of HCl. In this way the concentration of originally isoelectric gelatin was, therefore, in every case 0.5 per cent. The second portion was treated in the same way except that before adding the acid the gelatin was kept for 1 hour at 45°C. This was done to melt part of the submicroscopic pieces of jelly assumed to exist in the solution and thus to increase the concentration of the isolated ions and molecules and to diminish the relative quantity of solid submicroscopic particles capable of occluding water and thereby causing the high viscosity characteristic of gelatin solutions. After this second portion of the stock solution of isoelectric gelatin had been kept for 1 hour at 45°C. it was rapidly cooled to 20°, the HCl was added in the way described for the first portion and the solutions were put into collodion bags to measure the osmotic pressure. Each collodion bag contained about 50 cc. of gelatin solution. The temperature now remained constant

at 20°C. for both sets of experiments. It was noticeable from the first that the osmotic pressure of the gelatin solution which had been kept for 1 hour at 45° and which was therefore supposed to have melted into smaller particles was higher than that of the gelatin solution not previously heated. Fig. 7 shows the result after 22 hours. The maximum osmotic pressure was, for the gelatin solution that had been previously heated, 200 mm. H_2O, while it was only 170 mm. for the other gelatin solution not previously heated to 45°C.

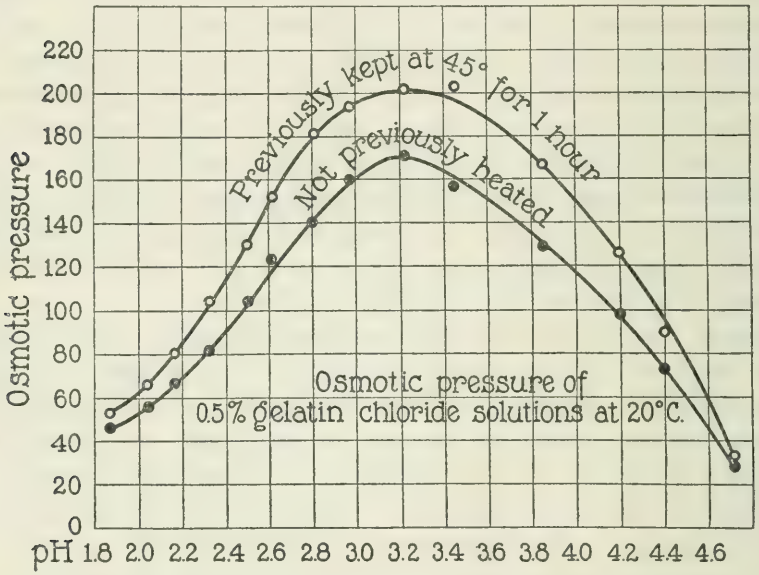

FIG. 7. Showing that the osmotic pressure of a solution of gelatin chloride which has been previously heated to 45°C. for 1 hour and then rapidly cooled to 20°C. is higher than the osmotic pressure of the same solution of gelatin chloride not previously heated.

Then the viscosities were determined at 20° and they gave the opposite result (Fig. 8), the viscosities being considerably higher in the solutions not previously heated to 45° than in the solutions previously heated. This experiment then confirms our expectation that there exists a reciprocal relation between the viscosity of protein solutions and their osmotic pressure, inasmuch as a transformation of solid submicroscopic particles of jelly into isolated protein ions

and molecules diminishes the viscosity but increases the osmotic pressure of the gelatin solution.

As far as the quantitative relations are concerned, the difference in viscosity (Fig. 8) is more striking than the difference in osmotic pressure (Fig. 7). The osmotic phenomena are a more complicated function of the change caused by the transformation of larger aggregates into simple ions, inasmuch as there exists a Donnan equilibrium not only between the gelatin solution and the outside watery solution

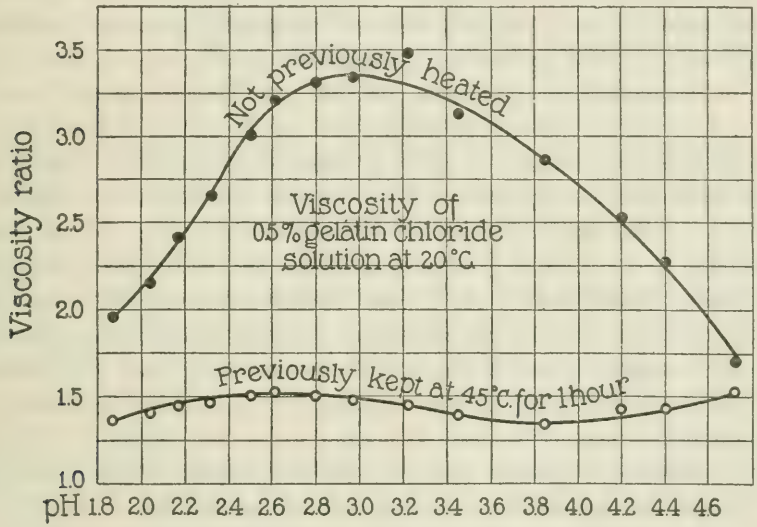

FIG. 8. Showing that the influence of previous heating on the viscosity of 0.5 per cent solutions of gelatin chloride is the reverse of the influence of the previous heating on osmotic pressure (Fig. 7).

but also between each solid particle of gelatin and the true gelatin solution surrounding it.

It was expected that when we put a 1 per cent solution of gelatin of, *e.g.*, pH 3.5, which had been kept for 1 hour at 45° and cooled to 20° into a 1 per cent solution of an identical gelatin chloride solution of pH 3.5, which had not been heated to 45° before being brought to 20°C., water would diffuse from the latter into the former solution. This experiment was carried out with a positive result.

These experiments support the idea expressed in the preceding paper that protein solutions are true solutions which may or may not contain solid particles of protein capable of swelling. In the case of gelatin solutions the formation of submicroscopic particles of solid jelly from isolated molecules or ions is a reversible process and this leads in this case to a reciprocal variation of osmotic pressure and viscosity of such solutions.

This probably explains a phenomenon which has puzzled the writer for a long time; namely, that the osmotic pressures of gelatin solutions of the same pH and concentration of originally isoelectric gelatin occasionally showed variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperature a gradual transformation of isolated molecules or ions into larger aggregates takes place, which must diminish the osmotic pressure but increase the viscosity. This source of variation was eliminated in the viscosity experiments in which the gelatin solution was always heated first to 45°C. and then as soon as this temperature was reached the solution was cooled to the temperature desired for the viscosity measurements. It is probable that the same uniformity of treatment is also required for the osmotic pressure experiments.

This reciprocal relation between osmotic pressure and viscosity exists probably also in the case of solutions of casein salts. Solutions of Na caseinate are less opaque than those of casein chloride (of the same concentration of originally isoelectric casein) which indicates that the Na caseinate solution contains more isolated casein ions and molecules and less submicroscopic solid particles than the solution of casein chloride.

The writer had already shown in a preceding paper that the maximal viscosity of a 1 per cent solution of casein chloride is higher than the viscosity of solutions of Na caseinate of equal concentration of originally isoelectric casein, while the osmotic pressures of solutions of the two salts show exactly the reverse relation, the maximal osmotic pressure of a 1 per cent solution of Na caseinate being almost 700 mm. H_2O while the maximal osmotic pressure of a 1 per cent solution of casein chloride is only about 200 mm.

The solutions of crystalline egg albumin seem to consist (at ordinary temperature and at not too high a concentration of albumin and of the hydrogen ions) exclusively or almost exclusively of isolated molecules or ions. Since the latter cannot diffuse through a collodion membrane they give rise to a Donnan equilibrium across the membrane and hence only the osmotic pressure of solutions of salts of crystalline egg albumin is influenced by electrolytes in the way demanded, while the viscosity shows such an influence only to a negligible degree.

3. Since in connection with viscosity we have assumed the existence of submicroscopic solid particles of jelly in gelatin solutions we must point out where our ideas agree and where they disagree with the speculations on the rôle of the degree of dispersion current in colloid chemistry. On the basis of these latter speculations it would be assumed that the osmotic pressure of a protein solution is determined directly by the concentration of the protein in solution according to van't Hoff's law. We assume the correctness of van't Hoff's law but the osmotic pressure is only in part determined by the gas pressure of the protein particles, the main source of the osmotic pressure being the unequal distribution of the crystalloidal ions on the opposite sides of the membrane due to the Donnan equilibrium. This has been overlooked by the believers in the dispersion theory. We agree with the believers in the dispersion theory that the melting down of larger aggregates of gelatin ions into isolated gelatin ions must raise the osmotic pressure but disagree in the explanation of this effect; since in our opinion the increase in the number of isolated protein ions must lead to an increase in the distribution of crystalloidal ions on the opposite sides of the membrane according to the Donnan equilibrium and this is the main reason why the osmotic pressure of a gelatin solution is increased when the solid submicroscopic particles of jelly are transformed into isolated ions.[3]

[3] If we wish to measure the true osmotic pressure of a protein solution, free from the complication of the Donnan equilibrium, we must do so at the isoelectric point of the protein. This condition was approximately fulfilled in the experiments of Sörensen (Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 262) on solutions of crystalline egg albumin, and of Hüfner and Gansser (Hüfner, G., and Gansser, E., *Arch. f. Physiol.*, 1907, 209) on solutions of hemoglobin.

On the other hand, the high viscosity of gelatin solutions, *e.g.*, that part of the viscosity which is due to the volume occupied by the solid particles of jelly, is entirely regulated by the Donnan equilibrium. The dispersion theory was not able to account for the influence of electrolytes on the viscosity and osmotic pressure of protein solutions while this influence is accounted for mathematically by the Donnan theory.

SUMMARY AND CONCLUSIONS.

1. These experiments confirm the conclusion that protein solutions are true solutions consisting of isolated ions and molecules, and that these solutions may or may not contain in addition solid submicroscopic particles capable of occluding water.

2. The typical influence of electrolytes on the osmotic pressure of protein solutions is due to the isolated protein ions since these alone are capable of causing a Donnan equilibrium across a membrane impermeable to the protein ions but permeable to most crystalloidal ions.

3. The similar influence of electrolytes on the viscosity of protein solutions is due to the submicroscopic solid protein particles capable of occluding water since the amount of water occluded by (or the amount of swelling of) these particles is regulated by the Donnan equilibrium.

4. These ideas are supported by the fact that the more the submicroscopic solid particles contained in a protein solution or suspension are transformed into isolated ions (*e.g.*, by keeping gelatin solution for 1 hour or more at 45°C.) the more the viscosity of the solution is diminished while the osmotic pressure is increased, and *vice versa*.

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THE INFLUENCE OF ELECTROLYTES ON THE SOLUTION AND PRECIPITATION OF CASEIN AND GELATIN.

By JACQUES LOEB and ROBERT F. LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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I. INTRODUCTION.

The modern concepts of colloid chemistry originated largely from a study of the precipitation of colloids by electrolytes, and this accounts, perhaps, for the fact that the tendency to form aggregates was considered the chief characteristic of colloids in solution. As a consequence it is assumed in the text-books of colloid chemistry that the ultimate units of a colloid in solution are not isolated molecules or ions but larger aggregates of molecules or ions, the so called micella (small crumbs) of Naegeli, which are supposed to be kept in a stable solution or suspension through forces of repulsion due to the fact that they are electrically charged. The precipitation of the particles by electrolytes is ascribed to a diminution of the charge of the micella through the adsorption of the ions of the electrolyte. It was noticed by Hardy that the active or precipitating ion of the electrolyte was always that ion which had the opposite sign of charge as the colloidal particle, and Picton and Linder had noticed that the precipitating efficiency of ions increased rapidly with their valency.[1]

The experiments published by one of us have led to results which are compatible with some but not all of the assumptions just enumerated. In the first place, experiments on the osmotic pressure and the viscosity of protein solutions have made it probable that the ultimate units in certain protein solutions, such as crystalline egg albumin, are essentially isolated protein ions or molecules, though such solutions may, in a secondary way, also contain aggregates of ions or molecules.[2]

[1] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 827; 1921–22, iv, 73, 97.

It was, secondly, found that proteins combine with acids and bases according to the stoichiometrical laws of classical chemistry, and that there is neither any need nor, in fact, any room for the assumption that the ions of electrolytes are adsorbed by the protein particles in solution.[3] Thirdly, the electrical charges of protein particles which occur in such solutions are due chiefly, if not exclusively, to either the electrolytic dissociation of protein salts or to membrane potentials caused by the Donnan equilibrium.[4] Fourthly, the influence of electrolytes on the P.D., the osmotic pressure, nad the viscosity of protein solutions[5] and the swelling of gels[6] can be explained quantitatively on the basis of the Donnan equilibrium. Under these circumstances it became necessary to find out whether or not the influence of electrolytes on the precipitation of proteins can be harmonized with these recent results.

All the workers who have studied the influence of electrolytes on the precipitation of colloids have come to the conclusion that there are two distinct groups of phenomena. In the one group the precipitation requires high concentrations of an electrolyte, while in the second, a low concentration of electrolyte suffices for precipitation. The difference between these two cases is so striking that it has been used for a classification of colloids. Thus, according to Zsigmondy, Noyes[7] discriminates between "colloidal solutions" and "colloidal suspensions;" the "colloidal solutions" being characterized by a high degree of viscosity, by a tendency to gelatinize, and by the fact that they are not easily precipitable by electrolytes; while the "colloidal suspensions" are, according to Noyes, characterized by a low order of viscosity, by a lack of tendency to gelatinize, and by the fact that they are easily precipitated by electrolytes.[8]

[3] Loeb, J., *Science*, 1920, lii, 449.

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667, 691, 827; 1921–22, iv, 73, 97.

[6] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

[7] Noyes, A. A., quoted in Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918, 29.

[8] Different authors have introduced different terms for these two groups of colloids, Perrin speaks of hydrophilic and hydrophobic colloids; Freundlich of lyophilic and lyophobic, and Wo. Ostwald of emulsoids and suspensoids.

Of all the characteristics for the two groups mentioned by Noyes, only one is essential, namely, the difference in the ease with which the two groups are precipitated by electrolytes, while the two other differences mentioned by him are only accidental. Thus a solution of crystalline egg albumin requires at ordinary temperature a high concentration of electrolyte for precipitation, although the viscosity of such a solution is low and although the solution has no tendency to gelatinize; while at a sufficiently high temperature, low concentrations of electrolytes will precipitate crystalline egg albumin though the viscosity of the solution is now high and though the solution has now a tendency to gelatinize. Hence only one of the differences mentioned in Noyes' definition remains; namely, the difference in the relative concentration required to precipitate colloids from their solution or their suspension.

The discrimination between the two types of precipitation according to whether high or low concentrations of electrolytes are required, is, however, essential and it becomes our first problem to account for this difference. We shall see that it is accompanied by another difference. When high concentrations of electrolytes are required for the precipitation of proteins the sign of charge of the protein particles is of little significance. Sulfates are much more effective for the salting out of gelatin or crystalline egg albumin from their watery solution than chlorides, regardless of whether the solution of the protein is at the isoelectric point or on the acid or alkaline side of it.

When low concentrations suffice for precipitation, the sign of charge of the protein ions becomes of paramount importance. On the acid side of the isoelectric point the active ion of the precipitating salt is the anion, while on the alkaline side it is the cation. It happens that this is also true for the Donnan effect and that in the Donnan effect also, low concentrations of electrolytes suffice for the depressing effect of a salt. This suggests the possibility that precipitation of the second group of colloids, *i.e.*, where low concentrations are required, is in some way connected with the Donnan equilibrium; and that Hardy's rule is only the consequence of this fact; while where high concentrations of electrolytes are required for precipitation the forces determining the process have no connection with the Donnan equilibrium.

In order to gain more definite information concerning the nature of the forces involved in the two cases it seemed advisable to supplement these investigations by a study of the mechanism of the solution of proteins. We have studied the mechanism of solution of granules of isoelectric casein in acid and in alkali and find that this mechanism is entirely different in the two cases. The solution of casein chloride is controlled by forces connected with the Donnan equilibrium; and it also happens that casein chloride when in solution can be precipitated by low concentrations of electrolytes; *e.g.*, M/8 NaCl. On the other hand, the solution of sodium caseinate is apparently controlled by the forces of chemical attraction between water and certain groups of the casein molecule; and it happens that very high concentrations of electrolytes, *e.g.*, $2\frac{1}{2}$ M NaCl or $3\frac{1}{4}$ M LiCl, may not be sufficient for precipitation.

II. The Precipitation and Solution of Casein Chloride.

1 per cent solutions of casein chloride of pH 2.2 were prepared in different concentrations of salts in water of about the same pH. That concentration was determined which causes an almost instantaneous complete precipitation of the protein from the originally milky solution so that the supernatant liquid became as clear as water. These concentrations were as follows:

NaCl	about M/8
$NaNO_3$	about M/8
$CaCl_2$	about M/8
Na trichloracetate	about M/16
Na_2SO_4	about M/32

Though the results are only semiquantitative, the validity of Hardy's rule and the valency effect are easily recognizable. Beside these two effects, some constitutional effects of the anion (such as the trichloracetate) may exist. It is also obvious that the concentrations of electrolytes required for instantaneous, complete precipitation of casein chloride are considerably lower than those required for the precipitation of Na caseinate from their watery solution, so that we can be sure that in the case of casein chloride we are dealing with a representative of the "suspension" group of colloids, in the sense of Noyes.

It can be shown that the solution of the casein chloride depends on forces regulated by the Donnan equilibrium and that the rule of Hardy is in this case at least only a consequence of this fact. This can be proven by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate,[9] but it was found that the mechanism of solution in this latter case is different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 cc. of water containing different quantities of different acids and kept at 24°C. In various intervals, *i.e.*, after 15, and 60 minutes, and 6, and 24 hours, the diameter of about 15 grains was measured with a micrometer in a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 1. The abscissæ are the logarithms of the concentrations of acid of the watery solution, the ordinates are the average diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH 2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Fig. 2 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest solubility for HCl and for H_3PO_4, *i.e.*, between pH of the outside solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

[9] Robertson, T. B., The physical chemistry of the proteins, London, Bombay, Calcutta, and Madras, 1918, 275 ff.

Figs. 1 and 2 show another fact; namely, that the rate of swelling is not the same in different acids. It is about the same in HCl and H_3PO_4 (for the same pH) but decidedly less in HNO_3 and still less in H_2SO_4 and trichloracetic acid. It was found that the rate of solution

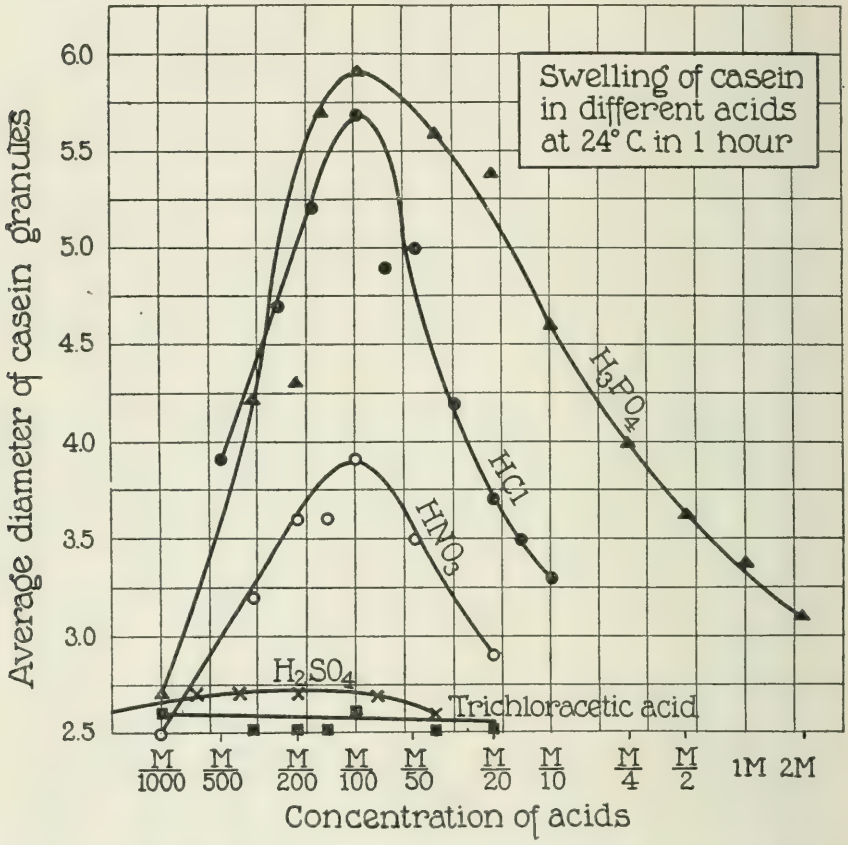

FIG. 1. Relative swelling of isoelectric granules of casein when put into acids of different concentrations, at 24°, after 1 hour. The hydrogen ion concentration of the casein particles is less than that of the outside solution on account of the Donnan equilibrium. The latter concentrations are plotted as abscissæ. The ordinates are the relative average diameters of the granules.

of casein in these different acids followed closely the rate of swelling. It took longer to dissolve casein in HNO_3 than it did in HCl (at 20°C.); and the casein was practically insoluble in H_2SO_4 and trichloracetic acid in 24 hours.

The rate of swelling is a function apparently not only of the Donnan equilibrium, but also of the force of cohesion between the particles. Procter and Wilson[6] have suggested that the rapid increase of swelling of solid gelatin with a rise in temperature is due to a corresponding

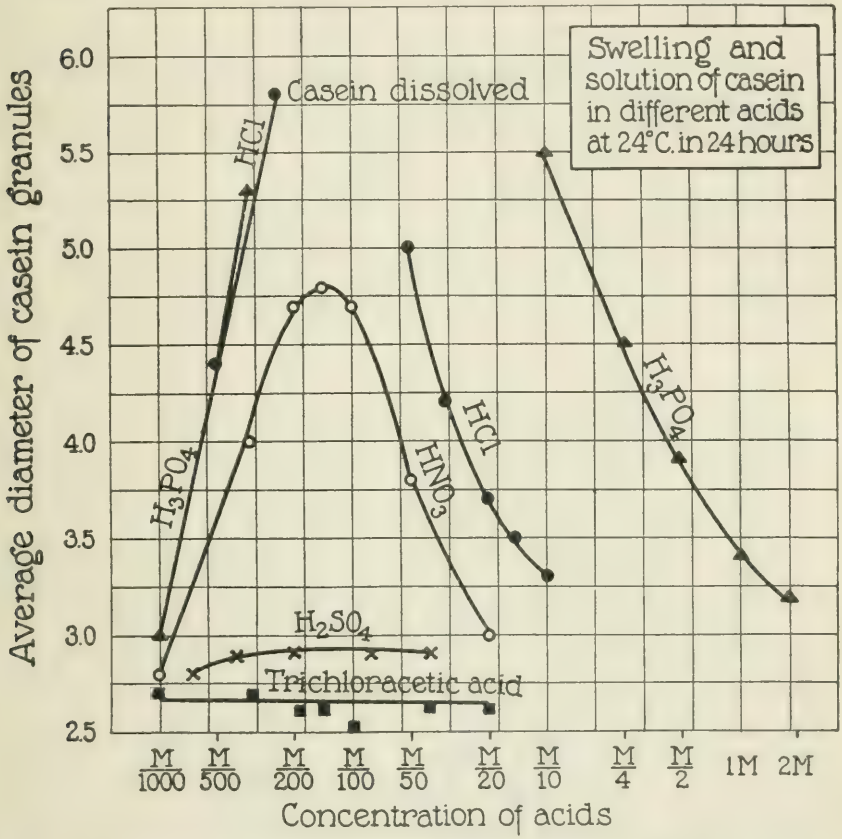

FIG. 2. Swelling of the same granules after 24 hours. Where the swelling exceeds a certain limit, between 5 and 6 in our scale, the particles are dissolved, thus showing that swelling is of importance in the mechanism of solution of casein chloride.

diminution of cohesion between the molecules of gelatin with rising temperature. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much less than the influence of the anion on the cohesion of casein particles. The

forces of cohesion in the case of casein sulfate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence no swelling (and as a consequence no solution) of solid casein is possible in H_2SO_4 or trichloracetic acid.

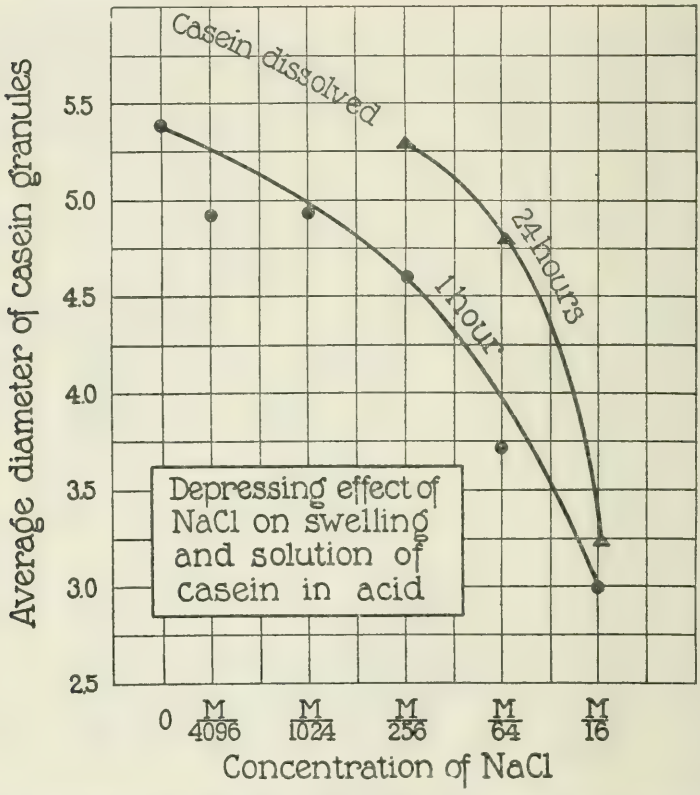

FIG. 3. Depression of swelling of casein particles in M/100 HCl by the addition of NaCl. A low concentration of acid suffices already for a noticeable depression of the swelling. The lower curve represents swelling after 1 hour; the upper curve swelling after 24 hours. Solution of the swollen particles occurs when the average diameter of the particles exceeds the value 5 to 6 in our scale.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling of solid gelatin.[6] Microscopic measurements of the influence of NaCl on the rate of swelling of individual grains of casein particles in M/100 HCl were made at 24°C. and the results plotted in Fig. 3.

The ordinates are the average diameters of the particles after 1 and 24 hours respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration of below M/256, but not in concentrations of NaCl higher than M/256.

That the solution of casein chloride is thus regulated to a large extent by the Donnan effect was ascertained also by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. 1 gm. of isoelectric powdered casein was put into 100 cc. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours respec-

TABLE I.

Amount of Casein Dissolved at 20°C in HCl of Different pH.

pH	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Mg. dissolved after 1 hour	42	55	86	249	265			348	408		547	538	401	366	272	219
Mg. dissolved after 22 hours	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

tively at 20°C. The supernatant liquid was removed and the sediment dried over night in an oven at about 100°C. Table I gives the result. The dry weight of 1 gm. of isoelectric casein was foundto be 0.870 gm. and this weight diminished by the dry weight of the sediment was the amount dissolved. Table I shows that, the rate of solution increases with diminishing pH from 4.4 to 2.18 where the solubility of casein chloride is a maximum; with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained. Solutions of 12.5 cc. of 0.1 N HCl in 100 cc. and containing 1 gm. of powdered, originally isoelectric casein were prepared in 0, M/2048, M/1024, to M/4 NaCl. The pH of a solution of 1 gm. casein in 100 cc. containing 12.5 cc. of 0.1 N HCl was 2.12 and this pH was the same in all solutions made up in NaCl.

The solution was kept at 20° for 16 hours and then allowed to settle for 24 hours at 20° in 100 cc. graduate cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in 2 cc. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table II gives the number of milligrams of casein which had gone into solution.

TABLE II.

	Concentration of NaCl.					
	M/2048	M/1024	M/512	M/256	M/128	M/64
Mg. dissolved.....	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes already a noticeable drop in the rate of solution. Thus M/1024 NaCl causes already a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 24°C.

These observations then indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure in the interstices between the casein ions. Procter and Wilson have shown that the application of Donnan's theory of membrane equilibrium accounts quanitatively for this swelling on the assumption that swelling is caused by the excess of the osmotic pressure inside the gel over that of the surrounding solution. As soon as the osmotic pressure in the gel exceeds the forces of cohesion between the casein ions of the gel the casein ions constituting the gel are separated.

The question then arises: How can the Donnan effect stabilize the particles of casein chloride in solution, and how can we explain the

precipitating effect of low concentrations of neutral salts? Let us assume that the ultimate particles in a solution of casein chloride of pH 2.2 are, (*a*) isolated casein ions, (*b*) isolated casein molecules, and (*c*) small casein aggregates or micella. The Donnan equilibrium furnishes two kinds of forces preventing that degree of coalescence of these particles which is required for precipitation; namely, the osmotic pressure and the membrane potentials. When isolated protein ions collide and remain attached to form a micellum, a Donnan equilibrium is established between the nascent micellum and the surrounding solution. The Donnan equilibrium demands that there be a higher concentration of electrolytes inside than outside and this difference in osmotic pressure leads to water being attracted into the micellum. The increase in hydrostatic pressure will force the protein molecules apart again and thus tends to prevent the formation of the micellum. Moreover, if micella exist in the casein chloride solution (aside from isolated casein ions and molecules) the coalescence of different micella into larger aggregates must be prevented by the potential difference between the micella and the surrounding solution. J. A. Wilson suggested in 1916[10] that the source of the charges might be the Donnan equilibrium, and one of us has recently shown that these potential differences between a gel and the surrounding solution, which Donnan's theory demands and which Wilson postulated, actually exist.[11] As a consequence of this P.D., the micella are charged and must repel each other according to the charge. This charge caused by the Donnan equilibrium is a minimum at the isoelectric point, rises with increasing hydrogen ion concentration, reaching a maximum, and diminishes again with a further increase in hydrogen ion concentration as shown in a preceding paper. The osmotic pressure and charge are also diminished by the addition of salt.

Hardy's rule that only that ion of a neutral salt is active in precipitation which has the opposite sign of charge as the colloidal ion, and that the efficiency of the ion increases with the valency is simply the expression of the Donnan effect, as is also the fact that very low concentrations of electrolytes suffice for precipitation. The reader will notice that it is unnecessary to assume that the ions

[10] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

[11] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

are adsorbed by the casein and that this adsorption of ions annihilates the electrical charges on the particles of casein.

Emulsions of oil drops in water are prevented from coalescing by their electrical charges (and not by osmotic forces). The investigations of Beutner[12] have shown that the P.D. at the boundary of water and substances immiscible in water is determined by an unequal distribution of crystalloidal ions between the two phases, and the writer ventures to suggest that this distribution may be regulated by Donnan's theory, owing to the fact that, *e.g.*, the oleic acid anion in oil cannot diffuse into the water. The similarity of the behavior of the P.D. between oil and water and between solid gelatin and water is rather striking. The depression of the charge on the particles through the addition of salt can in both cases be explained without the adsorption hypothesis.

III. The Precipitation and Solution of Na Caseinate.

When we prepare solutions of Na caseinate of pH 11.0 containing 1 gm. of originally isoelectric casein in 100 cc., we notice that it requires enormous concentrations of NaCl or LiCl to precipitate the casein. NaCl as concentrated as $2\frac{1}{2}$ M and LiCl as concentrated as $3\frac{1}{4}$ M were not able to precipitate Na caseinate from its solution, while M/8 NaCl was sufficient for instantaneous and complete precipitation of casein chloride from its solution. Hence Na caseinate belongs to the other group of colloidal solutions; namely, those which require high concentrations of electrolytes.

When a grain of isoelectric casein is put into a solution of NaOH Na caseinate is formed at the surface of the solid granule. This dissolves in water, as any crystalloidal substance does (*i.e.*, by the forces of residual valency), except for the accidental fact that the solution of Na caseinate seems to lower the surface tension of the watery solution at the interface, as is suggested by the rather violent spreading of the Na caseinate solution at the interface, which can be observed under the microscope. As a result projecting particles at the surface of the granule are torn away from the surface of the solid casein, collect-

[12] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

ing at a short distance, while the originally rough surface of the grain of casein becomes smooth. These phenomena of solution accompanied by spreading occur soon all over the surface. The small particles of casein carried away in the spreading motion are gradually completely dissolved and the result is a solution of Na caseinate of a high osmotic pressure, indicating that the solution consists to a large extent of isolated protein ions.

The mechanism of the solution of casein in NaOH is essentially that of the solution of a crystal except for the purely accidental and secondary phenomenon that the surface tension of the Na caseinate solution is considerably lower than that of a watery NaOH solution, and that thus the orderly process of solution noticeable in the case of crystals is disturbed by phenomena of spreading. The mechanism of solution of grains of solid casein in NaOH resembles the solution of sodium oleate in water, which is also accompanied by a lowering of the surface tension at the interface. This interference of the phenomena of spreading in the process of solution of Na caseinate may account for the fact that Robertson[9] was unable to confirm in this case the solution theory of Noyes and Whitney,[13] according to which there exists at the boundary of crystals and water a film of solution which is always saturated, the velocity of solution being determined by the rate of diffusion of the dissolved crystalloid out of this film into the body of the fluid. It is natural that this theory should be masked whenever the process of solution is accompanied by phenomena of spreading due to a lowering of surface tension at the interface. The forces dragging the particles of Na caseinate into solution are to all appearances those forces of residual valency between certain groups of the molecule of Na caseinate and water which also cause the solution of crystalloids in water, while the forces dragging the particles of casein chloride into water are forces which have their origin in the Donnan equilibrium. A quotation from Langmuir will illustrate the rôle which active groups of a complex molecule may play in the process of true solution.

"Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity

[13] Noyes, A. A., and Whitney, W. R., *Z. physik. Chem.*, 1897, xxiii, 689.

for each other. When oleic acid is placed on water the acid spreads upon the water because by so doing *the COOH can dissolve in the water* without separating the hydrocarbon chains from each other.

"When the surface on which the acid spreads is sufficiently large the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.

"Oils which do not contain active groups, as for example pure paraffin oil, do not spread upon the surface of water."[14]

That the solution of isoelectric casein in NaOH has no connection with the Donnan equilibrium is also evidenced by the fact that there is no point in the concentration of NaOH where further increase in the concentration of NaOH lowers the rate of solution, as would be the case if the Donnan equilibrium influenced this phenomenon.

The stability of solutions of this kind is guaranteed by the forces of chemical attraction between certain groups of the molecule of Na caseinate and water; and the precipitation by electrolytes is due to a diminution of these forces.

IV. Precipitation and Solution of Gelatin in Water.

Solutions of gelatin in water require enormous concentrations of salts for precipitation and it is very probable that the forces causing solution are the forces of residual valency discussed in the case of the solution of Na caseinate. These cases have no connection with the Donnan equilibrium, and this is borne out by the well known fact that solutions of gelatin are always more readily salted out by sulfates than by chlorides, regardless of the pH of the gelatin solution. This is illustrated by Table III.

0.8 per cent solutions of gelatin were prepared at three different pH; namely, 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and 6.4 to 7.0 (Na gelatinate). The purpose was to find the molecular concentration of different salts, namely, $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$, KCl, and $MgCl_2$, required for precipitation. Table III shows that regardless of the pH the sulfates are better precipitants than the chlorides. There is, therefore, no definite relation between the sign of charge of the colloidal particles and of the precipitating ion.

[14] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1850.

Precipitation experiments give no insight into the reason why sulfates are superior to chlorides in salting out; and an attempt was made to get an answer by experiments on the solution of gelatin. Such experiments gave the result that salts increase the solubility of isoelectric gelatin, and the more, the higher their concentration until finally a concentration is reached where the reverse effect is noticed. This reversal occurs at a comparatively low concentration in the case of the sulfates, while in the case of salts like $CaCl_2$ or $MgCl_2$ it does not seem to occur at all in the limit of the concentrations tried. The experiments were carried on at a temperature of 35°C.

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in previous articles, and 0.8 gm. was put into 100 cc. of each of a series of solutions of NaCl, $CaCl_2$, or Na_2SO_4 varying in concentration from M/4096 to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension at 35°C. was measured. We consider this time the reciprocal of the solubility. The ordinates in Fig. 4 are the solution times (*i.e.*, the reciprocal of the solubility) of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that NaCl and still more $CaCl_2$ increase the solubility (or diminish the solution time) of isoelectric gelatin in water, and the more, the higher the concentration of the salts. There exists a striking discontinuity in the Na_2SO_4 curve. As long as the concentration of Na_2SO_4 is below M/32 it increases the solubility of gelatin, and the more so, the higher the concentration. When, however, the concentration

TABLE III.

Minimal Molar Concentrations Required to Precipitate 0.8 Per Cent Solutions of Gelatin.

pH of gelatin solution.	Approximate molecular concentration of salt required for precipitation.				
	$(NH_4)_2SO_4$	Na_2SO_4	$MgSO_4$	KCl	$MgCl_2$
4.7	15/16 M	6/8 M	10/8 M	>3 M	>3 M
3.8 (gelatin chloride)	13/16 M	5/8 M	7/8 M	3 M	>3 M
6.4 to 7.0 (Na gelatinate)	16/16 M	7/8 M	9/8 M	>3 M	>3 M

of Na_2SO_4 is above M/32, a further increase in the concentration of Na_2SO_4 diminishes the solubility of gelatin, and the more so, the higher the concentration of Na_2SO_4. $(NH_4)_2SO_4$ acts in the same way. We now understand why it is that we cannot precipitate solutions of isoelectric gelatin with KCl or $MgCl_2$ in concentrations

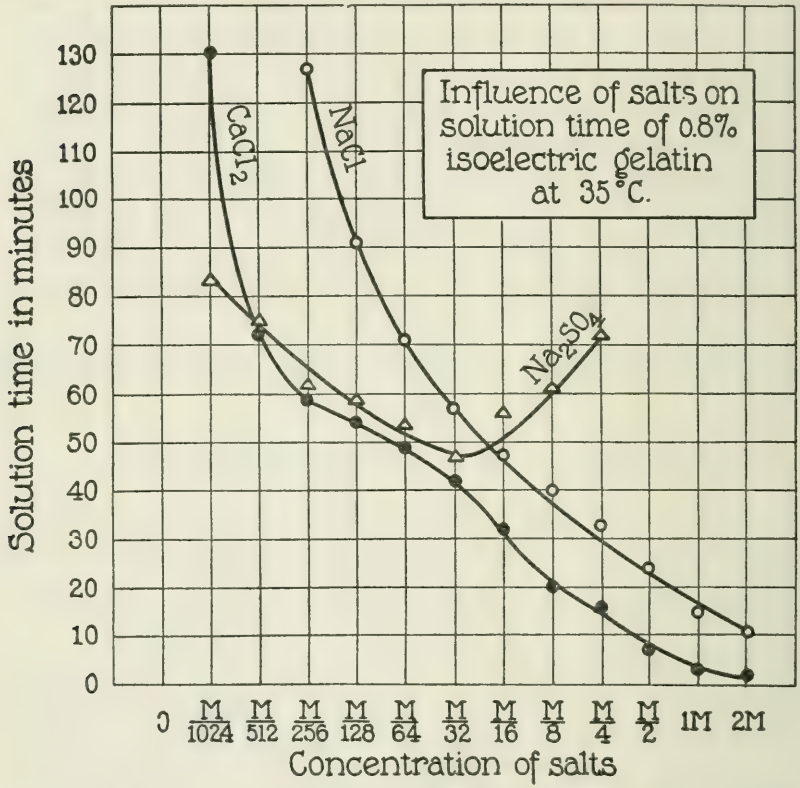

FIG. 4. Influence of salts on the time required to completely dissolve 0.8 gm. of powdered isoelectric gelatin in 100 cc. of salt solution of different concentrations at 35°C. Abscissæ are the concentrations of the salt; ordinates, the time required for complete solution.

up to 3 M (see Table III) while we can precipitate such solutions with sulfates but only at concentrations above M/2; since our curve shows that at such high concentrations of sulfates the solubility of isoelectric gelatin in Na_2SO_4 becomes less than in pure water.

While isoelectric gelatin is only sparingly soluble, gelatin salts

are highly soluble. 0.8 gm. of powdered gelatin of pH of about 3.3 dissolves very rapidly in 100 cc. HCl of the same pH at 35°C. The addition of NaCl or $CaCl_2$ no longer increases the solubility, except when the concentration of $CaCl_2$ becomes greater than M/16. Na_2SO_4

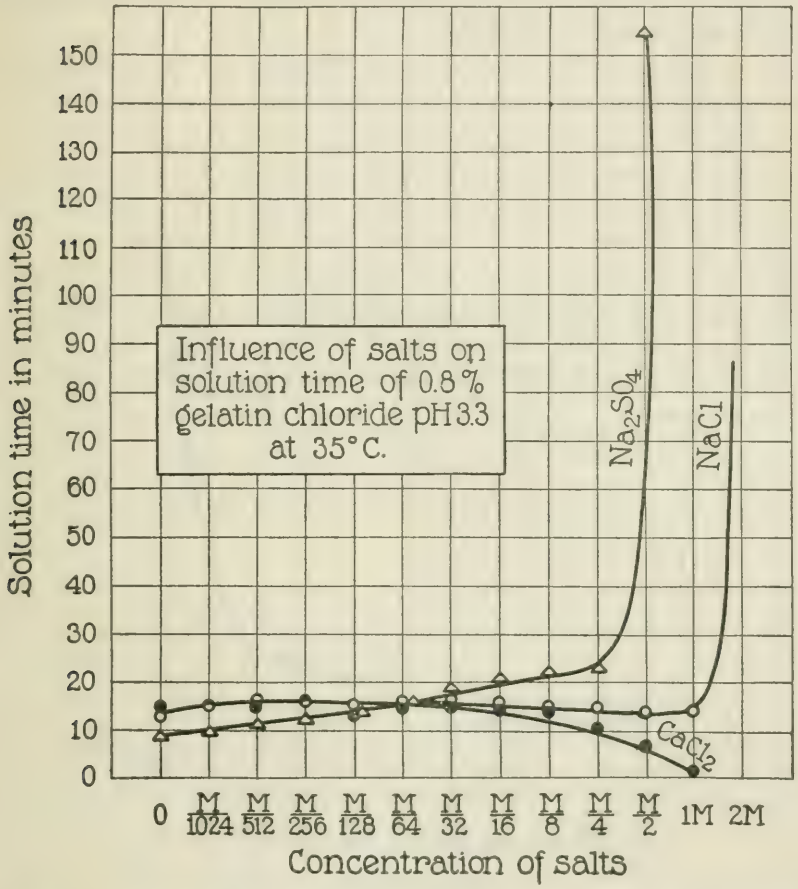

FIG. 5. Influence of salts on time required to dissolve 0.8 gm. of powdered gelatin chloride of pH 3.3 at 35°C.

abruptly diminishes the solubility at a concentration above M/4 and NaCl above a concentration of 1 M (Fig. 5).

Fig. 6 shows the influence of the three salts on the solution time of Na gelatinate of pH 10.5. Na_2SO_4 diminishes the solubility abruptly

at a concentration above M/8, while both NaCl and $CaCl_2$ increase the solubility of Na gelatinate, NaCl in concentrations above M/2, and $CaCl_2$ in concentrations above M/16.

In all three cases, therefore, is the solubility of gelatin diminished by sulfates, but only exceptionally by chlorides. This explains the results contained in Table III.

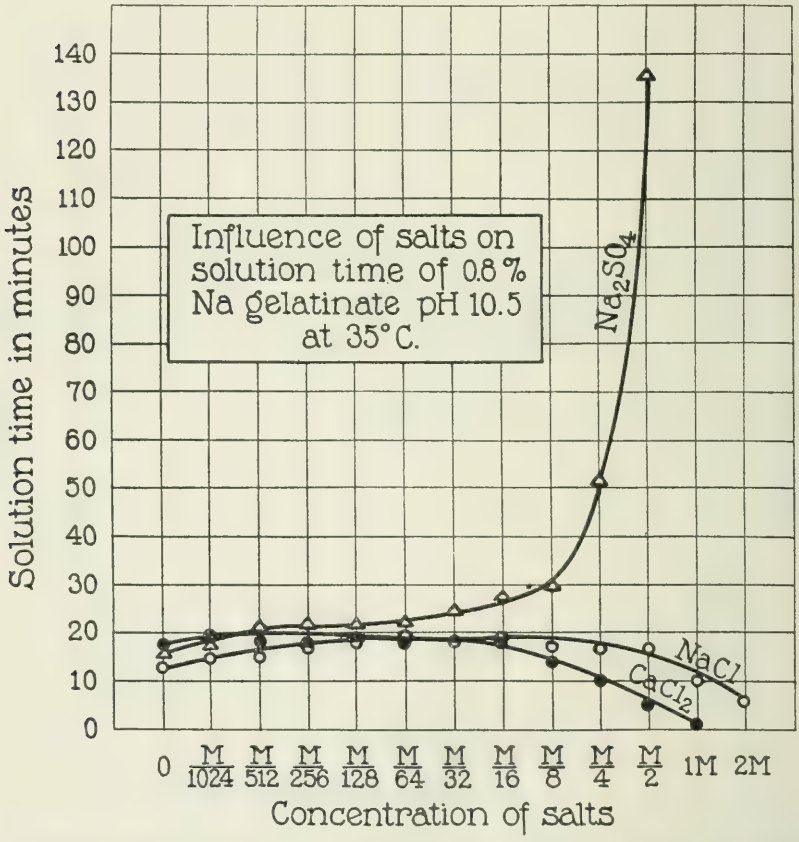

FIG. 6. Influence of salts on the time required to dissolve 0.8 gm. of powdered Na gelatinate of pH 10.5 at 35°C.

We have seen that addition of little acid to isoelectric gelatin increases the osmotic pressure, viscosity, P.D., and swelling with increasing concentration, while beyond a certain pH the addition of more acid has a depressing effect. This is characteristic of the

Donnan effect. It was of interest to find out whether such a maximum followed by a drop existed in the influence of acid on the solubility of gelatin, but this is not the case at least between pH 4.7 and 1.0. Measurements of the dry weight of gelatin dissolved in a certain time at different pH, showed that the amount of gelatin dissolved increases with the hydrogen ion concentration. This corroborates the conclusion that the solution (and precipitation) of gelatin in water is not influenced by forces governed by the Donnan equilibrium and does therefore not show the characteristics of colloidal behavior.

These experiments also contradict the suggestion that the solution of solid gelatin is necessarily preceded by swelling and that swelling and solution are continuous processes. The contradiction lies in the fact that swelling in acid reaches a maximum at pH of about 3.0 and then diminishes upon further increase in hydrogen ion concentration, while the rate of solution of solid gelatin granules continues to increase steadily when the hydrogen ion concentration increases beyond pH of 3.0. The mechanism of swelling and the mechanism of solution of solid gelatin in water are determined by forces of an entirely different character; the swelling by osmotic pressure, and the solution by the secondary valency forces responsible for the solution of crystalloids.

V. Solubility and Viscosity of Gelatin Solutions.

We assumed in a preceding paper that the increase in the viscosity of gelatin solutions on standing is due to the gradual formation of larger aggregates from originally isolated gelatin molecules or gelatin ions. When we melt a solid gel of gelatin by rapidly heating it to 45°C. and cooling it rapidly to 20° there is produced a true solution containing isolated gelatin molecules or gelatin ions side by side with submicroscopic pieces of solid jelly. On standing two opposite processes are constantly going on in such a mixture; *i.e.*, the solution of these aggregates into isolated molecules or ions and the reverse process; namely, that of the formation of new aggregates by the union of formerly isolated gelatin molecules or gelatin ions. When the velocity of aggregate formation prevails over the velocity of the solution of aggregates, the viscosity of the gelatin solution will increase on standing; when the velocity of solution of aggregates

prevails over the velocity of formation, the viscosity of the gelatin solution will diminish on standing. All agencies which accelerate the rate of solution of solid gelatin should counteract the aggregate formation and the rise of viscosity of a gelatin solution on standing; while all agencies which diminish the rate of solution of solid gelatin should increase the rise of viscosity of the gelatin solution on standing.

We have seen that powdered isoelectric gelatin is dissolved the more rapidly, the more HCl we add, and that there is no maximum followed by a drop when a certain hydrogen ion concentration is exceeded. Hence we should expect that the lower the pH of a gelatin solution containing the same concentration of originally isoelectric gelatin, the smaller the increase of the viscosity of the solution on standing. In a preceding paper we have already given curves showing that this is the case and that there is no Donnan effect noticeable in this case.[15]

We have seen in Fig. 5 that Na_2SO_4 commences to diminish noticeably the rate of solution of solid gelatin chloride as soon as the concentration of Na_2SO_4 exceeds M/64, while $CaCl_2$ commences to have the opposite effect as soon as the concentration of $CaCl_2$ exceeds M/4.

We prepared gelatin chloride solutions of pH 3.4 containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The solutions were made up in various concentrations of Na_2SO_4 and $CaCl_2$. The solutions were rapidly heated to 45°, rapidly cooled to 20°C., and kept at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and in intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at 20° was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises gradually but very slowly (uppermost curve in Fig. 7) and the rate of increase of viscosity on standing is not materially altered in M/512 Na_2SO_4 and only little in M/128 Na_2SO_4. In M/32 Na_2SO_4 the viscosity increases more rapidly on standing, in M/8 Na_2SO_4 still more rapidly, and in M/2 Na_2SO_4 very sharply. This is exactly what we should expect since the Na_2SO_4 causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of

[15] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 97.

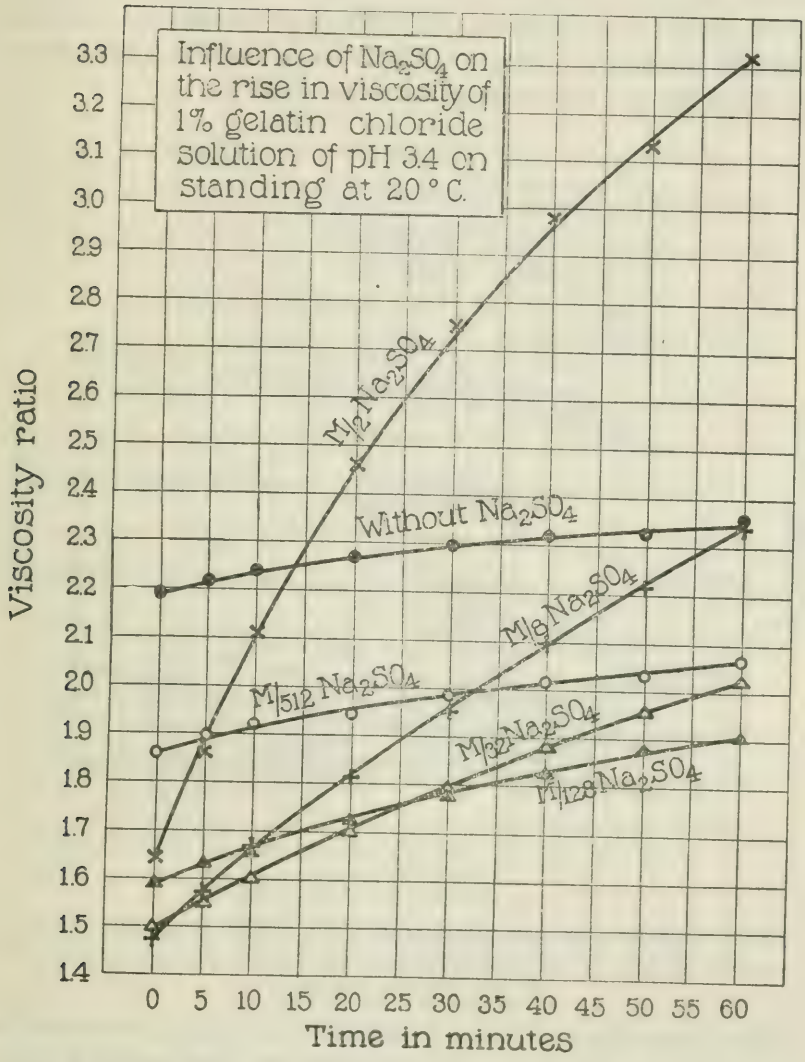
Influence of Na_2SO_4 on the rise in viscosity of 1% gelatin chloride solution of pH 3.4 on standing at 20° C.
Viscosity ratio
Time in minutes
$M/2\ Na_2SO_4$
Without Na_2SO_4
$M/8\ Na_2SO_4$
$M/512\ Na_2SO_4$
$M/32\ Na_2SO_4$
$M/128\ Na_2SO_4$

FIG. 7. Showing that concentrations of Na_2SO_4 of M/32 and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Na_2SO_4 is above M/64. In such solutions the rate of solution of micella will be less and less, and since new micella are constantly formed at 20°C., the viscosity will rise more rapidly on standing when the solution contains Na_2SO_4 in concentrations above M/64 than when the solution contains no or less Na_2SO_4.

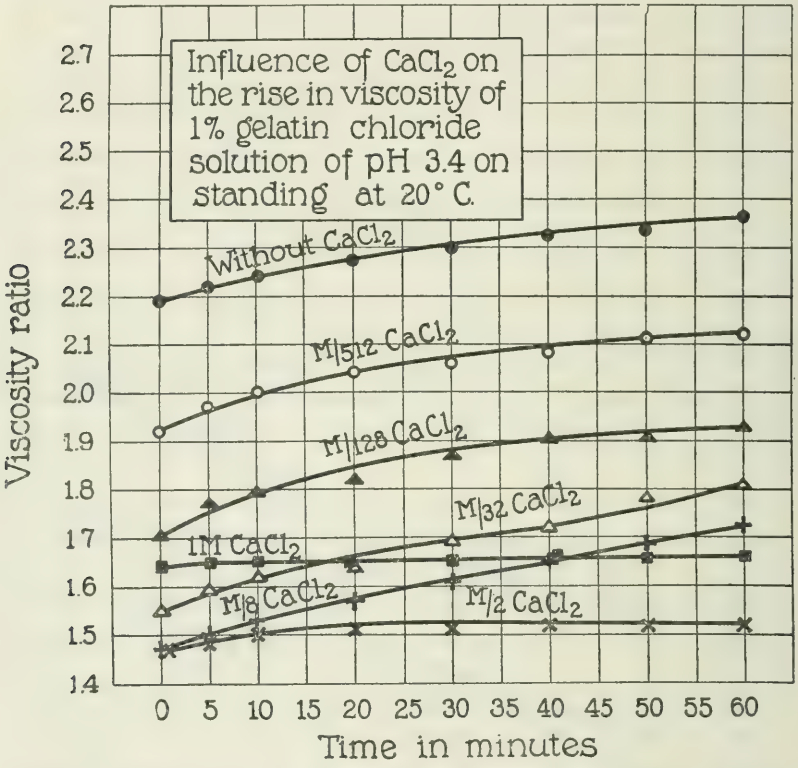

FIG. 8. Showing that concentrations of $CaCl_2$ of M/2 or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Fig. 8 shows that $CaCl_2$ in concentrations up to M/8 does not alter the increase in viscosity of gelatin chloride solution on standing but that the viscosity of gelatin chloride of pH 3.4 does no longer increase on standing when the concentration of $CaCl_2$ is M/2 or 1 M. In this concentration $CaCl_2$ causes a slight increase in the rate of solution of gelatin chloride.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 M. Above this concentration it causes coagulation and the viscosity can no longer be measured. Hence NaCl in concentrations up to 1 M should

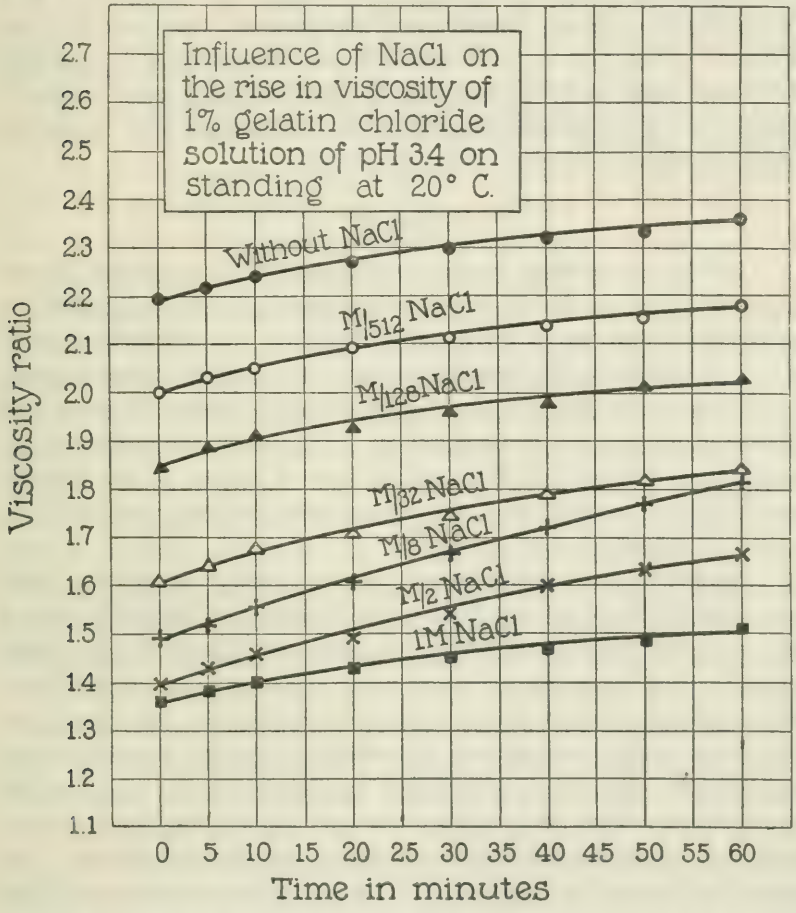

FIG. 9. Showing that NaCl solutions up to a concentration of 1 M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Fig. 9 shows that this is correct.

These experiments corroborate the idea that gelatin solution

are true solutions consisting of isolated gelatin ions and molecules which may contain, in addition, submicroscopic aggregates of gelatin molecules and ions. The formation of new micella and a solution of these already formed occur constantly. When the rate of formation of such micella exceeds the rate of the solution, the viscosity of a gelatin solution rises on standing and the rise is in proportion with the difference in the rate of the formation and solution of these micella. The reader must bear in mind that the micella in the case of gelatin solutions are submicroscopic particles of a reversible jelly of gelatin and not particles of an irreversible precipitate.

SUMMARY.

1. Colloids have been divided into two groups according to the ease with which their solutions or suspensions are precipitated by electrolytes. One group (hydrophilic colloids), *e.g.*, solutions of gelatin or crystalline egg albumin in water, requires high concentrations of electrolytes for this purpose, while the other group (hydrophobic colloids) requires low concentrations. In the latter group the precipitating ion of the salt has the opposite sign of charge as the colloidal particle (Hardy's rule), while no such relation exists in the precipitation of colloids of the first group.

2. The influence of electrolytes on the solubility of solid Na caseinate, which belongs to the first group (hydrophilic colloids), and of solid casein chloride which belongs to the second group (hydrophobic colloids), was investigated and it was found that the forces determining the solution are entirely different in the two cases. The forces which cause the hydrophobic casein chloride to go into solution are forces regulated by the Donnan equilibrium; namely, the swelling of particles. As soon as the swelling of a solid particle of casein chloride exceeds a certain limit it is dissolved. The forces which cause the hydrophilic Na caseinate to go into solution are of a different character and may be those of residual valency. Swelling plays no rôle in this case, and the solubility of Na caseinate is not regulated by the Donnan equilibrium.

3. The stability of solutions of casein chloride (requiring low concentrations of electrolytes for precipitation) is due, first, to the osmotic pressure generated through the Donnan equilibrium between

the casein ions tending to form an aggregate, whereby the protein ions of the nascent micellum are forced apart again; and second, to the potential difference between the surface of a micellum and the surrounding solution (also regulated by the Donnan equilibrium) which prevents the further coalescence of micella already formed. This latter consequence of the Donnan effect had already been suggested by J. A. Wilson.

4. The precipitation of this group of hydrophobic colloids by salts is due to the diminution or annihilation of the osmotic pressure and the P.D. just discussed. Since low concentrations of electrolytes suffice for the depression of the swelling and P.D. of the micella, it is clear why low concentrations of electrolytes suffice for the precipitation of hydrophobic colloids, such as casein chloride.

5. This also explains why only that ion of the precipitating salt is active in the precipitation of hydrophobic colloids which has the opposite sign of charge as the colloidal ion, since this is always the case in the Donnan effect. Hardy's rule is, therefore, at least in the precipitation of casein chloride, only a consequence of the Donnan effect.

6. For the salting out of hydrophilic colloids, like gelatin, from watery solution, sulfates are more efficient than chlorides regardless of the pH of the gelatin solution. Solution experiments lead to the result that while $CaCl_2$ or NaCl increase the solubility of isoelectric gelatin in water, and the more, the higher the concentration of the salt, Na_2SO_4 increases the solubility of isoelectric gelatin in low concentrations, but when the concentration of Na_2SO_4 exceeds M/32 it diminishes the solubility of isoelectric gelatin the more, the higher the concentration. The reason for this difference in the action of the two salts is not yet clear.

7. There is neither any necessity nor any room for the assumption that the precipitation of proteins is due to the adsorption of the ions of the precipitating salt by the colloid.

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THE ORIGIN OF THE POTENTIAL DIFFERENCES RESPONSIBLE FOR ANOMALOUS OSMOSIS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 19, 1921.)

I. Potential Differences in Collodion Membranes Coated with Protein.

In a series of papers[1] the writer has published the results of experiments showing the peculiar influence of electrolytes on the rate of diffusion of water through collodion membranes. When a collodion membrane separates a solution of an electrolyte (of not too high a concentration) from pure warer, the water diffuses from the side of pure water to the solution side, but the initial rate at which this diffusion occurs does not obey van't Hoff's law. The initial rate of diffusion was measured by the rise of level of liquid in a glass tube inserted through a rubber stopper in a closed collodion bag containing the solution, the collodion bag dipping into a beaker filled with pure water. When the membrane was merely a collodion membrane, the following rules expressed the influence of electrolytes on the initial rate of diffusion of water through the membrane from the water side to the side of the solution.

1. Water diffuses into the solution of an electrolyte through a collodion membrane as if the particles of water were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte in solution with a force increasing with the valency.

2. In the case of certain electrolytes the "attracting force" of the anion for water increraes at first more rapidly with increasing concentration than the "repelling force" of the cation, until a point is reached where with a further increase in concentration the "repelling force" of the cation increases more rapidly than the "attractive force" of the anion. Finally, a concentration is reached where the

[1] Loeb, J., *Science*, 1921, liii, 77; *J. Gen. Physiol.*, 1918–19, i, 717; 1919–20, ii, 173, 387, 577.

"attracting" and "repelling" forces balance each other and from then on the "attractive" force of the solution increases with the concentration, apparently in accordance with van't Hoff's law.

When, however, the collodion membrane has received a coating of a protein, *e.g.*, gelatin, the hydrogen ion concentration influences the

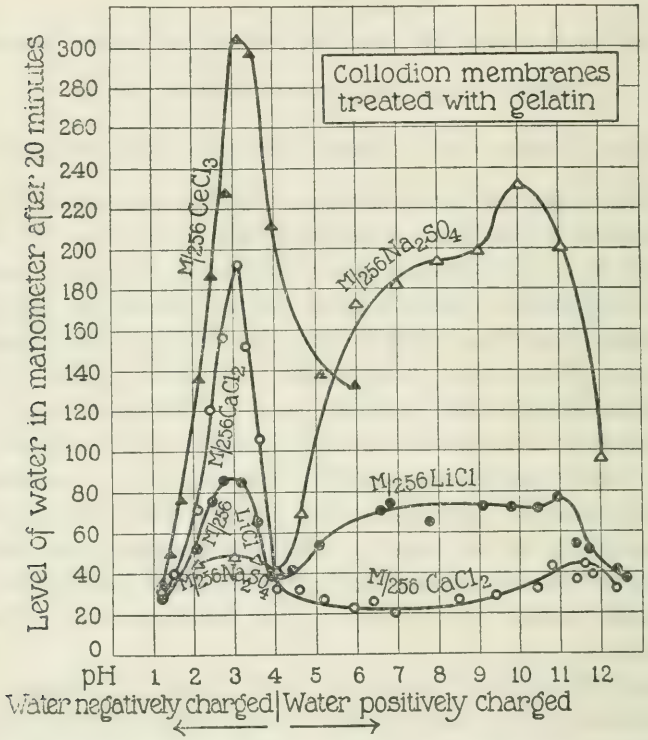

FIG. 1. Influence of hydrogen ion concentration on the initial rate of diffusion of liquid from a solution of HNO_3 or NaOH respectively through a gelatin-coated membrane of collodion into a M/256 solution of LiCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4 of the same hydrogen ion concentration. Abscissæ are the initial pH; ordinates, the rise of level of liquid in manometer connected with the salt solution, after 20 minutes.

initial rate of diffusion of water from pure water into the solution in a still more complicated way (Fig. 1). The procedure in these experiments was as follows: Both the outside solution as well as the inside solution had the same hydrogen ion concentration,

containing the same concentration of HNO_3 or NaOH respectively. In addition the inside solution (*i.e.*, the solution inside the collodion bag which was connected with the manometer) contained a M/256 solution of one of the following four salts: $CeCl_3$, $CaCl_2$, LiCl, and Na_2SO_4. The "attraction" of these M/256 salt solutions for water was therefore tested at different hydrogen ion concentrations. It is obvious from Fig. 1 that the "attractive" force of one and the same salt solution for water (measured by the initial rate of diffusion of water from the outside into the salt solution) varied considerably with the hydrogen ion concentration of the solution. (The hydrogen ion concentration is expressed by Sörensen's logarithmic symbol pH; *i.e.*, the log of the concentration with the minus sign omitted.) The abscissæ in the curve are the initial pH of the solutions, while the ordinates are the rise in level of the watery liquid in the glass tubes after 20 minutes at 24°C.

Fig. 1 shows that somewhere between pH 4.0 and 5.0 a reversal of the sign of charge of the water particles occurs. At pH below 4.0 the water is negatively charged, at pH above 5.0, it is positively charged. This change coincides with a change in the nature of the charge of the gelatin ion. At a pH of 4.0 or below the gelatin forms gelatin nitrate (in the presence of HNO_3) and hence the gelatin ion is a cation. The water, being negative, has the opposite sign of charge as the gelatin ion. At a pH of 5.0 or above the gelatin forms metal gelatinate, Na gelatinate, Ca gelatinate, etc., and the gelatin ion is negatively charged. The water, being positively charged, has again the opposite charge as the gelatin ion. The exact turning point, the isoelectric point of gelatin, is at a pH 4.7. In a preceding paper[2] the writer has been able to show that if other proteins than gelatin are used to give the collodion membrane a coating of a protein, the reversal of the sign of charge of the water varies with the isoelectric point of the protein used.

If we return to Fig. 1 we notice that where the water is negatively charged it is "attracted" by the cation of the salt used and "repelled" by the anion with a force increasing with the valency of the ion. The attractive force increases from Li to Ca to Ce, and diminishes from

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

Cl to SO_4. Where the water is positively charged the reverse order of efficiency exists.

The fact that the same concentration of the salts, namely, M/256, was used, has no essential influence on the results. Similar results were obtained when the salt solutions had the osmotic pressure of a M/64 solution of cane sugar.

Those who have studied phenomena of abnormal osmosis through membranes, Girard,[3] Bernstein,[4] Bartell and his collaborators,[5] and Freundlich[6] have reached the conclusion that potential differences on the opposite sides of the membrane are responsible for these anomalies in diffusion. The fact that the sign of charge varies in these experiments with the sign of charge of the protein ion gives a favorable point of attack for the investigation of the origin of the potential differences. In order to simplify the experiments they were confined to pH from 4.6 to pH 1.9; *i.e.*, to that region where the water diffuses as if it were negatively electrified. The method of procedure was as follows. M/256 solutions of one of the four salts mentioned were prepared in different concentrations of HNO_3 and put into collodion bags of about 50 cc. volume, which were lined by a film of gelatin on the inside. These bags were closed with a perforated rubber stopper through which was pushed a glass tube with a diameter of about 2 mm. to indicate the rise of liquid. The collodion bags were put into beakers containing 350 cc. of the same concentration of HNO_3 as that inside the bag. Fig. 2 gives the rise of the level of liquid in the manometer in one set of experiments after 1 hour at 24°C. The abscissæ are the initial pH of the liquid which was the same inside and outside, the ordinates are the rise of the level of liquid after 1 hour. It is obvious in this case that the curves have a minimum near the isoelectric point of gelatin, that the rate of diffusion of water into the solution rises

[3] Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le méchanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

[4] Bernstein, J., Elektrobiologie, Braunschweig, 1912.

[5] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.

[6] Freundlich, H., *Kolloid. Z.*, 1916, xviii, 11.

with the increasing hydrogen ion concentration reaching a maximum at pH 3.6 or 3.0 respectively, and that the rate of diffusion falls again with a further increase in the hydrogen ion concentration. M/256 Na_2SO_4 shows little "attraction" for water, and M/128 cane sugar practically none.

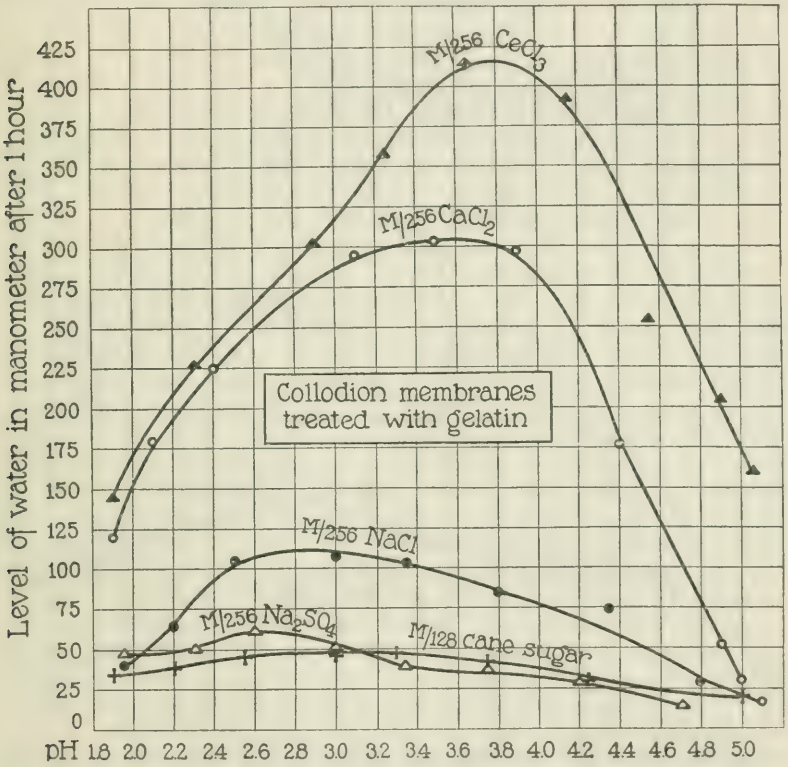

FIG. 2. Influence of hydrogen ion concentration on initial rate of diffusion of liquid from a solution of HNO_3 through a gelatin-coated collodion membrane into a M/128 solution of cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4 of the same hydrogen ion concentration. Abscissæ are the pH; ordinates, rise of level of liquid in manometer connected with the salt solution, after 1 hour. Water negatively charged.

The writer has recently measured the potential differences between the inside salt solution and the outside solution at the end of an hour with the aid of a Compton electrometer (calomel electrodes with

saturated KCl solution) and obtained a set of curves presented in Fig. 3. At this time some of the salt had diffused from the inside into the outside solution. The P.D. curves in Fig. 3 resemble the osmotic curves in Fig. 2 sufficiently to suspect a connection

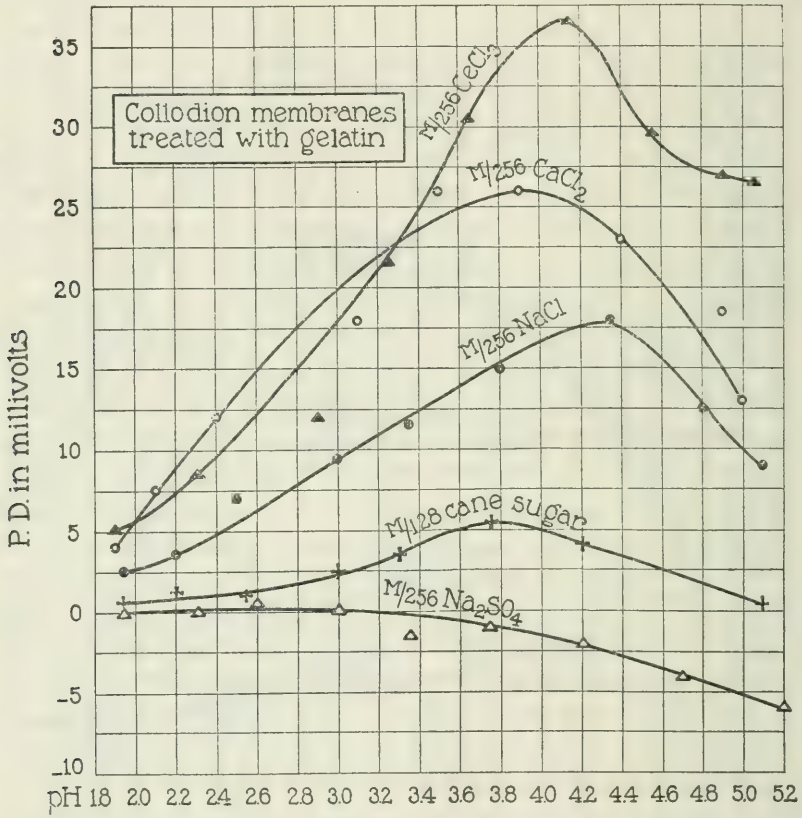

FIG. 3. Potential difference between salt solutions and external solution in preceding experiment after 1 hour of diffusion. Abscissæ are the pH at beginning of experiment; ordinates, P.D. at the end of the experiment. Attention is called to a certain similarity of the curves in Figs. 2 and 3.

between the two, so that we may assume that the P.D. between the opposite sides of the membrane is the main driving force which (for low concentrations of electrolytes) moves the water from the water side to the solution side of the membrane.

The next question was: What is the origin of the P.D.? It occurred to the writer that a measurement of the hydrogen ion concentration inside and outside after 1 hour might possibly throw a light on this problem. The results of these measurements are plotted in Fig. 4. The reader will remember that at the beginning the hydrogen ion concentration was the same inside and outside. Yet after 1 hour

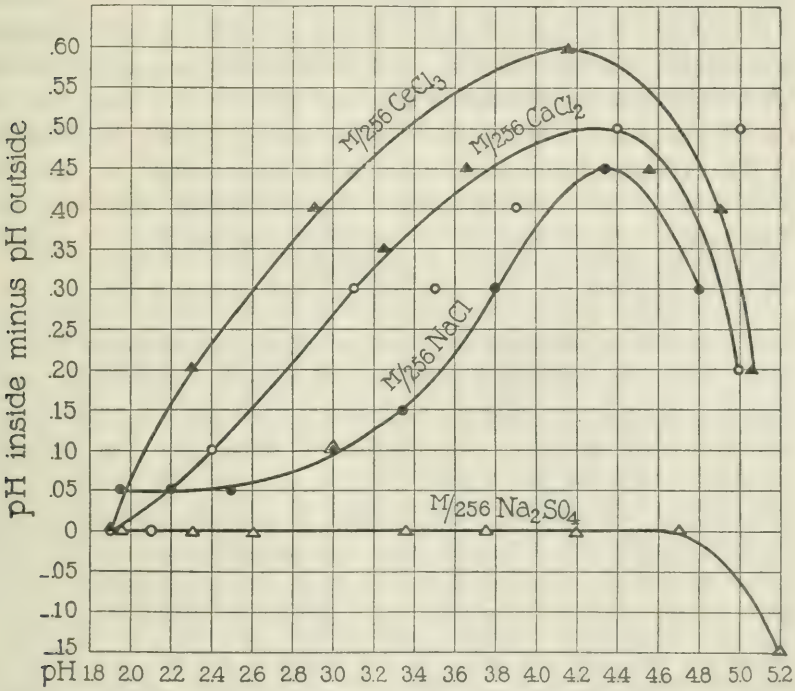

FIG. 4. Abscissæ are the initial pH; ordinates, the values (pH inside minus pH outside) in preceding experiment. Attention is called to the similarity of the curves in Figs. 3 and 4.

(and in fact much earlier) it is obvious that some of the acid has diffused from the inside into the outside solution so that the hydrogen ion concentration is higher outside than inside the collodion bag containing the salt solution.

This then leads to the result that when we separate identical acid solutions by a collodion membrane coated with a film of gelatin, and put inside the collodion bag a solution of a neutral salt with a mono-

valent anion (*e.g.*, Cl), acid is driven from the side where the salt is added to the side originally free from salt. This difference in the pH inside minus pH outside gives rise to a potential difference which was discussed in a preceding paper.[7] In this paper it was shown that when we separate a gelatin chloride solution from a solution of HCl (without gelatin) both having at the beginning the same hydrogen ion concentration, acid is forced from the gelatin solution into the outside solution. The same happens when solid jelly of gelatin chloride is separated from a HCl solution. In this case there arises a P.D. between the two phases; and the writer has been able to show that this P.D. can be calculated with a good degree of accuracy from the difference of the hydrogen ion concentration inside and outside, on the basis of Nernst's well known formula. By multiplying the value (pH inside minus pH outside) by 58 we get the value for the P.D. actually observed at 18°C. in terms of millivolts.[7]

This unequal distribution of acid inside and outside is due to a peculiar membrane equilibrium the theory of which was developed by Donnan.[8] Our new experiments show that such an equilibrium condition is produced also between a gelatin membrane and a solution of HCl free from gelatin. The coating of gelatin on the collodion membrane behaves like a solid jelly of gelatin, the gelatin being transformed into gelatin chloride when in contact with HCl. The Donnan equilibrium demands that the concentration of free HCl inside the gelatin membrane be less than the concentration of free HCl of the solution bounding the membrane. This gives rise to the P.D. The value of pH inside minus pH outside is diminished when a neutral salt is added—in accordance with Donnan's theory—and the fact that a salt is added in our diffusion experiments on one side of the membrane but not on the other is the cause of the fact that the pH of the acid solution containing the salt solution (inside solution) becomes higher than the pH of the solution containing no or less salt (outside solution). The reader will find the experiments proving this in the writer's paper referred to.[7]

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[8] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

A comparison of Figs. 3 and 4 shows that the two sets of curves, that for the P.D. (Fig. 3) and for the values pH inside minus pH outside (Fig. 4) when plotted over the initial pH as abscissæ, have certain features in common. Both sets of curves have a minimum at or near the isoelectric point of gelatin (pH 4.7). They both rise to a maximum at or not far from pH 4.2, and then drop again when the initial pH of the solution drops further. This parallelism suggests that this form of the P.D. curve is determined by the differences in the hydrogen ion concentration produced on the opposite sides of the membrane in conformity with Donnan's theory of membrane equilibrium.

II. Potential Differences in Collodion Membranes Free from Protein.

The experiments just described were repeated with collodion membranes which had not been treated with a protein. The difference between the P.D. in this case (Fig. 5) and the P.D. observed in the case of collodion membranes coated with a film of gelatin (Fig. 3) is striking. The method of experimentation was the same in the case of the collodion bags free from gelatin as in the experiments of the preceding chapter. The initial concentration of HNO_3 was always the same inside and outside. The inside solution, however, contained in addition to the acid a solution of M/256 of one of the four salts, $CeCl_3$, $CaCl_2$, NaCl, and Na_2SO_4. After 1 hour the P.D. inside and outside was measured.

We notice that the P.D. is no longer a minimum at or near pH = 4.0 and we can therefore be certain that this feature of the curves in Fig. 3 was due to the gelatin. The curves in Figs. 3 and 5 have, however, one feature in common; namely, that the P.D. increases with the increasing valency of the cation and diminishes with the increasing valency of the anion. This feature is, therefore, not a specific function of the film of gelatin.

Since the water is positively charged even in the presence of acid when the membrane consists of collodion free from protein, it is to be expected that only the Na_2SO_4 solution should attract the water, while no such attraction should occur in the case of M/256 $CeCl_3$ or M/256 $CaCl_2$. Fig. 6 shows that this is true.

The question then arises: How can we account for this valency effect of the ion with the same sign of charge as that of the particle of water? The answer seems to be that we are dealing here with diffusion potentials which are independent of the membrane. This was proved

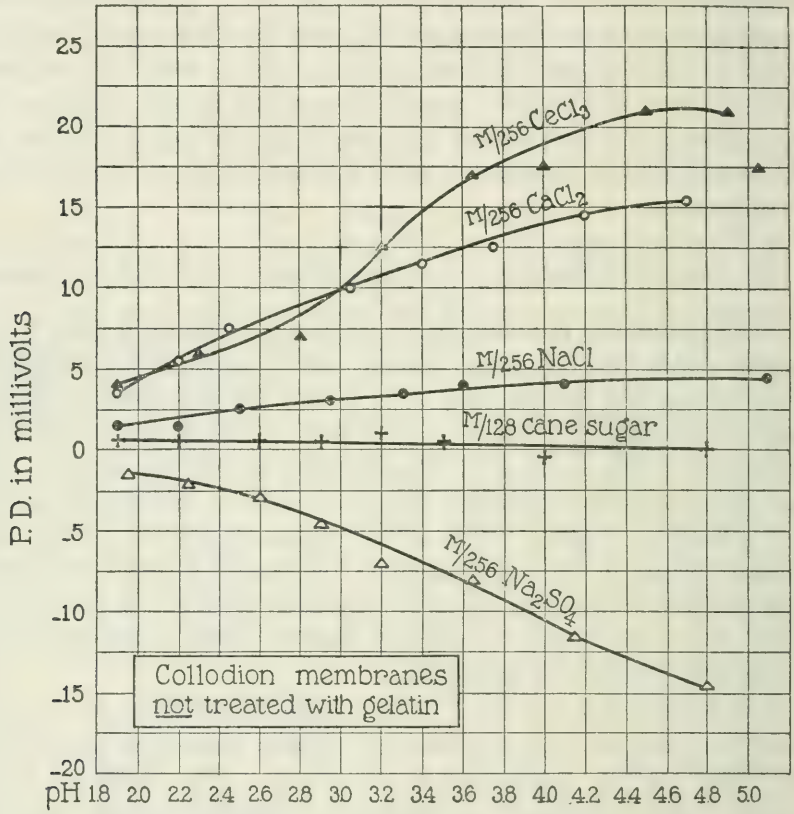

FIG. 5. Influence of neutral salts on the P.D. across collodion membranes not treated with gelatin. Otherwise same experiment as in Fig. 3. Notice difference between curves in Figs. 5 and 3, revealing the share of the protein in the origin of the P.D.

in the following way. Two beakers were filled with identical solutions of HNO_3, the HNO_3 solution in one of the beakers being dissolved in M/256 $CeCl_3$ or $CaCl_2$ or NaCl or Na_2SO_4, while the HNO_3 in the other was dissolved in pure water. The pH was identical in

the two solutions. They were connected by a bent glass tube, filled with a pure HNO_3 solution, of the same pH as that in the two beakers. Into each beaker was dipped a calomel electrode with saturated KCl solution and the diffusion potential was ascertained with the aid of a Compton electrometer. Fig. 7 gives the P.D. A comparison between Figs. 5 and 7 shows that the curves representing the P.D. in the two cases are very similar.

If we now return to a discussion of the curves in Figs. 1 and 2, representing the influence of the pH on the attraction of a M/256 solution of a neutral salt for water through a collodion membrane impregnated

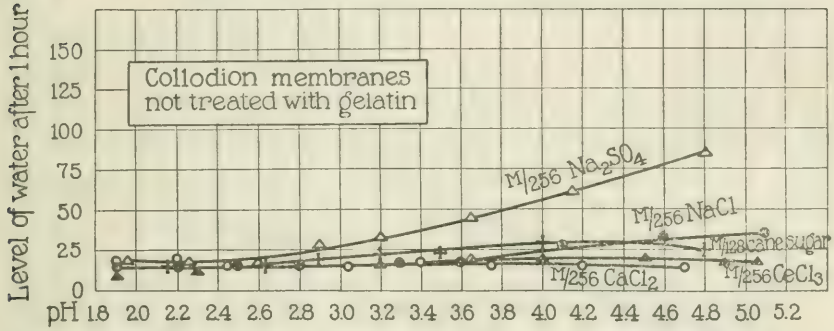

FIG. 6. Influence of M/128 cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, and Na_2SO_4 on the initial rate of diffusion of water through collodion membranes not treated with gelatin. Otherwise the experiment is the same as in Fig. 2. Abscissæ are the pH; ordinates, rise of level of water in salt solution after 1 hour. Only Na_2SO_4 attracts water to a noticeable amount since the latter is positively charged when diffusing through the membrane.

with gelatin, we can say that these curves resemble the curves in Fig. 3 for the P.D. on the opposite sides of the membrane.

Figs. 4 and 7 show that the P.D. curves in Fig. 3 have a double source. One is connected with the influence of the gelatin layer on the unequal distribution of the acid on the opposite sides of the membrane, which finds its explanation probably in the Donnan effect. The other source of the P.D. seems to be the diffusion potentials as shown in Fig. 7.

These diffusion potentials which exist regardless of the presence or absence of a membrane and regardless of the nature of the membrane seem to be responsible for the fact that the ions with opposite sign

of charge as that of the water "attract" the water with a force increasing with the valency of the ion, while the Donnan effect seems to be

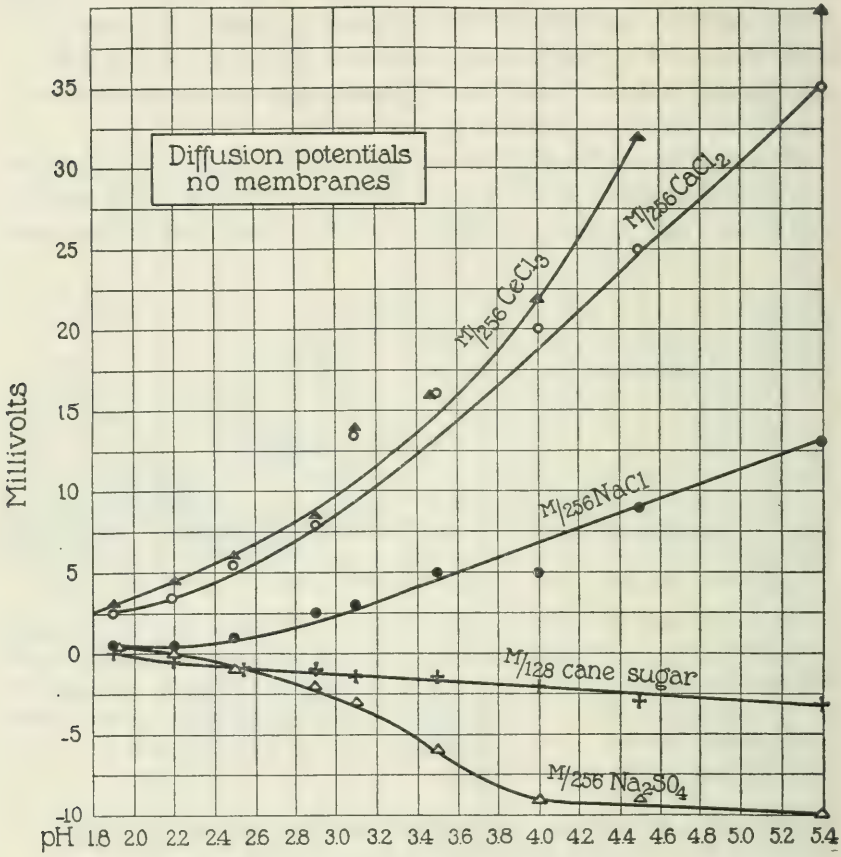

FIG. 7. Diffusion potentials between solutions of M/128 cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, and Na_2SO_4 in various concentrations of HNO_3 and solutions free from these salts but of the same concentrations of HNO_3. The salt solutions and the solutions free from salt had always the same pH. Abscissæ are the pH; ordinates, diffusion potentials. Attention is called to the resemblance between the curves in Fig. 7 and those in Fig. 5.

responsible for the fact that when the collodion membrane is impregnated with a protein the rate of diffusion of the negatively charged water is a minimum at the isoelectric point of the protein, increasing

with a diminishing pH until a maximum is reached at a pH of about 4.2, and diminishing with a further diminution of the pH.

These experiments then lead to the result that the anomalous osmosis discussed in this paper is determined by a potential difference between the opposite sides of the membrane, and that this P.D. seems to have a double source; namely, the Donnan equilibrium and diffusion potentials.

In addition to these two sources of P.D. between the opposite sides of the membrane there may be others, as, *e.g.*, the Coehn effect due to the difference in the dielectric constant between liquid and membrane. If such a P.D. plays a rôle in our experiments it can be only of minor importance.

The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.

SUMMARY AND CONCLUSION.

1. Collodion bags coated with gelatin on the inside were filled with a M/256 solution of neutral salt (*e.g.*, NaCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4) made up in various concentrations of HNO_3 (varying from N/50,000 to N/100). Each collodion bag was put into an HNO_3 solution of the same concentration as that inside the bag but containing no salt. In this case water diffuses from the outside solution (containing no salt) into the inside solution (containing the salt) with a relative initial velocity which can be expressed by the following rules: (*a*) Water diffuses into the salt solution as if the particles of water were negatively charged and as if they were attracted by the cation and repelled by the anion of the salt with a force increasing with the valency of the ion. (*b*) The initial rate of the diffusion of water is a minimum at the hydrogen ion concentration of about N/50,000 HCl (pH 4.7, which is the point at which gelatin is not ionized), rises with increasing hydrogen ion concentration until it reaches a maximum and then diminishes again with a further rise in the initial hydrogen ion concentration.

2. The potential differences between the salt solution and the outside solution (originally free from salt) were measured after the

diffusion had been going on for 1 hour; and when these values were plotted as ordinates over the original pH as abscissæ, the curves obtained were found to be similar to the osmotic rate curves. This confirms the view expressed by Girard, Bernstein, Bartell, and Freundlich that these cases of anomalous osmosis are in reality cases of electrical endosmose where the driving force is a P.D. between the opposite sides of the membrane.

3. The question arose as to the origin of these P. D. and it was found that the P.D. has apparently a double origin. Certain features of the P.D. curve, such as the rise and fall with varying pH, seem to be the consequence of a Donnan equilibrium which leads to some of the free HNO_3 being forced from the solution containing salt into the outside solution containing no (or less) salt. This difference of the concentration of HNO_3, on the opposite sides of the membrane leads to a P.D. which in conformity with Nernst's theory of concentration cells should be equal to 58 $\times$ (pH inside minus pH outside) millivolts at 18°C. The curves of the values of (pH inside minus pH outside) when plotted as ordinates over the original pH as abscissæ lead to curves resembling those for the P. D. in regard to location of minimum and maximum.

4. A second source of the P.D. seems to be diffusion potentials, which exist even if no membranes are present and which seem to be responsible for the fact that the rate of diffusion of negatively charged water into the salt solution increases with the valency of the cation and diminishes with the valency of the anion of the salt.

5. The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.

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STUDIES OF ACIDOSIS.

XVII. The Normal and Abnormal Variations in the Acid-Base Balance of the Blood.

By DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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The possible variations in the acid-base balance of the blood may be stated as follows: the blood bicarbonate may be high, low, or normal, and in each of these conditions the pH may be high, low or normal. There are as thus classified nine theoretically possible conditions. Only one of them is normal, that in which both bicarbonate and pH are within the normal limits. At the time of the first paper of this series (Van Slyke and Cullen, 1917) only two of the abnormal possibilities had come under clinical observation, that in which bicarbonate is low, and pH normal (compensated acidosis), and that in which bicarbonate is very low, and pH also low (uncompensated acidosis). Now, however, as the result of the recent work of Y. Henderson and Haggard, of Scott, of Milroy, of Collip, of Davies, Haldane, and Kennaway, of Grant and Goldman, of Peters and Barr, and of others, it is known that the other six abnormal possibilities can be produced experimentally, and that at least some of them occur clinically. For this reason it has seemed desirable to enlarge the view presented in our former paper in order to include within it these conditions.

The Normal and Abnormal Ranges of Hydrion Concentration of the Blood Plasma and Other Extracellular Fluids.—The average normal hydrion concentration of the blood plasma lies at or near the slightly alkaline point $H^+ = 4 \times 10^{-8}$, or pH = 7.4. This figure was estimated on the basis of material then available by L. J. Henderson in 1909, and has been confirmed by Lundsgaard (1912), Hasselbalch, and other subsequent investigators, utilizing the gas chain method. Parsons (1917) working with especial precautions showed that the

actual pH value determined is that of the plasma, and that the pH of venous blood in a given individual is normally only 0.02 below that of arterial blood. The maximum normal range of variation of blood reaction in different individuals appears to be indicated by pH 7.30 to 7.50. It is possible that when errors of technique are more completely excluded this range will become still narrower. For a given individual in the resting condition the data of Parsons and of Hasselbalch indicate that the pH variation may be only a few units in the second decimal place.

Under extreme abnormal conditions the pH may fall as low as 6.95, but before this point is reached it appears that coma occurs, and, from the fact that lower pH values have not been observed, it is doubtful that further decrease is compatible with life. This was the lowest point observed by Hasselbalch and Lundsgaard (1912) in rabbits killed by prolonged breathing of air which contained CO_2. It was also the lowest point observed by Van Slyke, Austin, and Cullen (1920) in experiments on etherized dogs. A pH of 6.95 was in one instance determined electrometrically by Cullen (unpublished) in the blood of a nephritic man in coma a few hours before death.

By voluntary deep breathing, on the other hand, carbonic acid may be blown off until the blood alkalinity rises to a pH of 7.7 or 7.8 (Davies, Haldane, and Kennaway, 1920; Collip and Backus, 1920), at which point, however, symptoms of tetany appear (Grant and Goldman, 1920). It therefore appears that the extreme range of reaction compatible with life lies approximately between pH 7.0 and 7.8, and that the normal range is within limits no greater than pH 7.3 to 7.5, and possibly somewhat narrower.

Concerning the pH of the body fluids other than blood plasma our knowledge is limited, but such as it is it indicates that these fluids approximate the blood plasma closely in their reaction. (By body fluids are meant the fluids within the body proper; such are lymph, cerebrospinal fluid, transudates, exudates, but not secretions such as gastric juice or urine.) Parsons and Shearer (1920) found in the cerebrospinal fluid a pH normal for blood plasma. Cullen and Boots in this Hospital (unpublished data) have observed a similar pH in joint fluids. In other body fluids, data of Collip and Backus (1920) and of others have shown that the bicarbonate is normal for

blood plasma. As there is reason to believe that the CO_2 tension in these fluids approximates that of the arterial blood (Haggard and Henderson, 1919) it appears that a bicarbonate concentration normal for blood plasma indicates also a $[BHCO_3]:[H_2CO_3]$ ratio, and therefore a pH in these fluids normal for blood plasma (B is used to indicate any monovalent base, such as Na or K, $[BHCO_3]$ to indicate the bicarbonate concentration, and $[H_2CO_3]$ the concentration of free carbonic acid).

The Normal and Abnormal Ranges of the Blood Bicarbonate Concentration.—Peters and Barr (1921) have reviewed the data in the literature and concluded therefrom that in normal men the total CO_2 content of the whole blood under 40 mm. CO_2 tension at 38° falls between 43 and 55 volumes per cent. The CO_2 content of the plasma is about 8 volumes per cent higher (Joffe and Poulton, 1920). Of the total CO_2 approximately $\frac{19}{20}$ is in the form of bicarbonate (Van Slyke and Cullen, 1917). When the CO_2 tension is other than 40 mm. the normal $[BHCO_3]$ figures also change. For when the CO_2 tension is increased the $[H^+]$ is also increased, and the ratio [BA]: [HA] for hemoglobin and the other buffers is lowered, since it changes in accordance with the equation $\frac{[BA]}{[HA]} = \frac{K}{[H^+]}$. ([BA] = concentration of alkali salt of buffer acid, and [HA] = concentration of free buffer acid.) In consequence of the change base from the buffers other than bicarbonate is set free and combines with CO_2 to form $BHCO_3$. Rise of CO_2 tension therefore increases not only the $[H_2CO_3]$ but also the $[BHCO_3]$ and *vice versa.* Consequently above and below 40 mm. CO_2 tension the normal limits of blood bicarbonate and total CO_2 content rise and fall as indicated by the two absorption curves of Fig. 1. (These curves are taken from Peters and Barr (1921), who plotted them to include an area containing all the apparently reliable CO_2 absorption curves of normal human blood in the literature.)

As shown by L. J. Henderson (1921) the variations in the acid-base balance of a given blood are indicated by the changes in any two of a number of interdependent variables, including the buffers other than bicarbonate, the plasma chloride, and the $[HbO_2]$: [Hb] ratio. In Fig. 1, for example, any point may be located by any two of four values; the total CO_2, the CO_2 tension, the pH, and the H_2CO_3.

Of such variables, however, the bicarbonate is of peculiar significance because, as a result of the unlimited supply of H_2CO_3, bicarbonate is the form taken by all bases in the blood not bound by acids other than carbonic. Consequently, as will be seen later, values of $[BHCO_3]$ taken together with those of another determining factor such as the pH, yield information concerning the available alkali which might be difficult to ascertain otherwise.

Representation of the Combined Variations in Blood Bicarbonate and Hydrion Concentration.—The blood conditions may be represented by a diagram of the type used by Haldane and others to show the "CO_2 absorption curves" of the blood, and recently further elaborated by Straub and Meier (1918) and by Haggard and Y. Henderson (1919) to show also the pH values.

If we draw a curve, expressing $[BHCO_3]$ values as ordinates, and $[H_2CO_3]$ values as abscissæ, the curve will be a slanting straight line for all points corresponding to any given $[BHCO_3] : [H_2CO_3]$ ratio, and the slant will be more or less steep according as the $[BHCO_3] : [H_2CO_3]$ ratio is great or small. But a constant $[BHCO_3] : [H_2CO_3]$ ratio indicates a constant pH (see equation of Hasselbalch below). Consequently, we are able by a series of straight, slanting lines on a diagram arranged as described to express all possible $[BHCO_3]:[H_2CO_3]$ ratios and pH values. In pure $NaHCO_3 - H_2CO_3$ solutions, the isohydrionic lines curve slightly, because the proportion of $NaHCO_3$ dissociated into Na^+ and HCO_3' increases slightly with dilution. In blood, where the Na^+ concentration is constant, the lines are practically straight. Their slope may be calculated from the equation of L. J. Henderson (1909), $[H^+] = K_1 \frac{[H_2CO_3]}{[BHCO_3]}$, or by the same equation in the logarithmic form used by Hasselbalch (1916) $pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]}$. $K_1 = \frac{K}{\lambda}$, K being the dissociation constant of carbonic acid, λ the degree of dissociation of $BHCO_3$ into B^+ and HCO_3'. pK_1 is the negative logarithm of K_1. The value of K_1 for blood was estimated by Haggard and Henderson (1919), from the data available in the literature, as 8×10^{-8}, for which the negative logarithm, and therefore the corresponding value of pK_1, is 6.10.

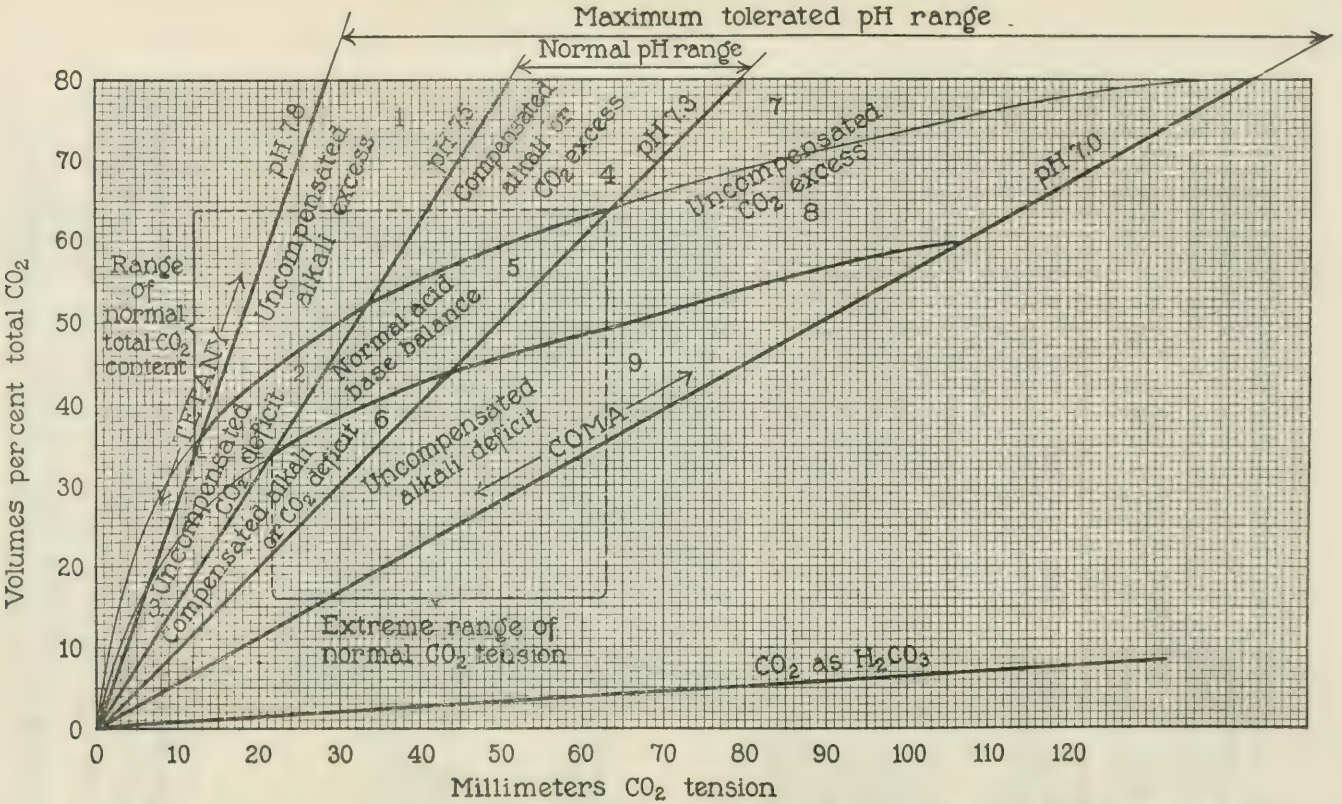

FIG. 1. Normal and abnormal variations of the $[BHCO_3]$, $[H_2CO_3]$, CO_2 tension, and pH in oxygenated human whole blood drawn from resting subjects at sea-level. The bicarbonate CO_2 at any point is obtained by subtracting from the total CO_2 the relatively small amount present as H_2CO_3 indicated by the slanting line near the bottom of the figure. (See discussion of Area 5.)

For blood in which the hemoglobin is completely reduced, Christiansen, Douglas, and Haldane (1914) have shown that the absorption curves are higher than in oxygenated blood by about 6 volumes per cent of CO_2 at ordinary CO_2 tensions.

The equation $pH = 6.10 + \log \frac{[BHCO_3]}{[H_2CO_3]}$ has accordingly been used in plotting the pH lines of Fig. 1 from which Figs. 2 and 3 are derived. That the value 6.10 will be subject to correction in the second decimal place as the result of further work appears probable but it is sufficiently accurate to serve our present purposes.

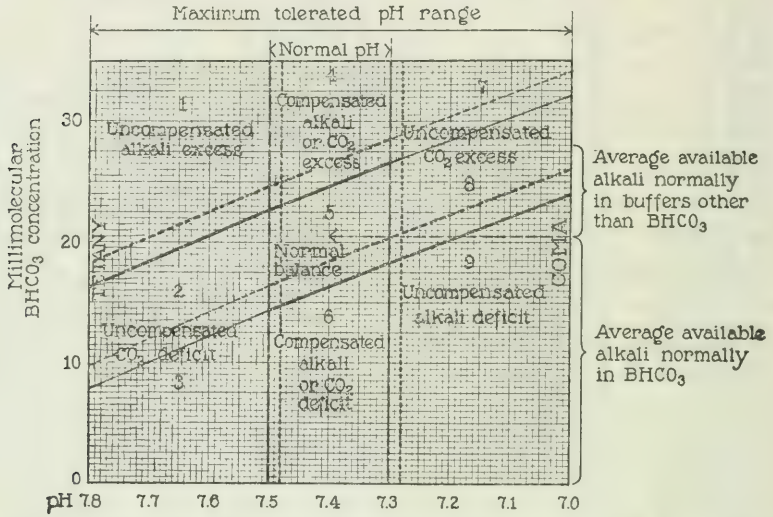

FIG. 2. Normal and abnormal variations of the $[BHCO_3]$ and pH values in arterial and venous human whole blood. The arterial conditions are indicated by the solid curves, venous by the broken curves. $[BHCO_3]$ and pH values are represented with rectangular coordinates, and the diagram, as compared with Fig. 1, is simplified by omitting $[H_2CO_3]$ and CO_2 tension values. $[BHCO_3]$ values are expressed in terms of millimolecular concentration (1 millimolecular $BHCO_3] = 2.24$ volumes per cent of bicarbonate CO_2).

For Fig. 1, since it was desired to use the customary form of CO_2 absorption curves, with total CO_2 values, $[BHCO_3 + H_2CO_3]$, as ordinates, and CO_2 tensions as abscissæ, the form of the equation used was $pH = 6.10 + \log \frac{(\text{total } CO_2) - (0.0672p)}{0.0672p}$, p being the CO_2 tension in mm. of mercury, 0.0672 the factor by which the tension is converted into terms of volumes per cent of CO_2 physically dissolved (as H_2CO_3) in the blood (Bohr, 1905).

The Combined Variations of Blood Bicarbonate and pH, and the Conditions Associated with Them.

As stated above, the possible conditions may be classified as those in which the blood bicarbonate is high, low, or normal, and combined with each of these may be a high, low, or normal pH, so that thus

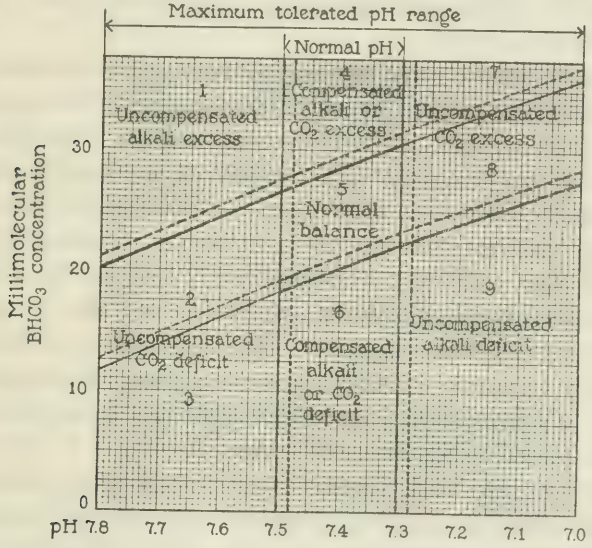

FIG. 3. Normal and abnormal variations of the [$BHCO_3$] and pH in serum or oxalate plasma. Arterial conditions are indicated by the solid curves, venous by the broken curves. The curves are 4 millimols higher than those of Fig. 2, since the $BHCO_3$ concentration in the plasma at any given pH is higher than that of the whole blood by approximately 10 volumes per cent of bicarbonate CO_2, or 4 millimols of $BHCO_3$ per liter. The venous bicarbonate curves are only half as far from the arterial as in the case of whole blood, since the data of Joffe and Poulton (1920), and of Smith, Means, and Woodwell (1921) indicate that the [$BHCO_3$] difference between venous and arterial blood is less in the plasma than in the cells.

classified there are nine possible different conditions of the acid-base balance. These conditions, represented by the nine areas shown in Fig. 1, are the following:

Area 1.—Uncompensated Alkali Excess.—In this condition the [$BHCO_3$] is increased above the normal without a parallel increase in

$[H_2CO_3]$. The result is an increase in the $[BHCO_3] : [H_2CO_3]$ ratio and therefore in pH. That is, the blood reaction becomes overalkaline.

The condition appears to occur after overdosing with sodium bicarbonate (Howland and Marriott, 1918; Harrop, 1919; Davies, Haldane, and Kennaway, 1920). It has also resulted from loss of gastric HCl caused by obstructing the pylorus and regularly washing out the stomach for some days (MacCallum, Lintz, Vermilye, Leggett, and Boas, 1920).

It is accompanied by an increase in alveolar CO_2 tension, due to a slowing of respiration in the apparent attempt of the organism to hold back sufficient CO_2 to restore to normal the overalkaline reaction. (If this compensation is accomplished the condition shifts to that represented by Area 4.) There is a moderate diuresis, and bicarbonate is excreted in the urine at a rate that may be several grams per hour (Davies, Haldane, and Kennaway). Ammonia almost completely disappears from the urine and the titratable acid may become a negative quantity. The cessation of acid excretion and its replacement by alkali excretion tend to reduce the $[BHCO_3]$ to normal, and bring the system back, probably through Area 4, to the normal condition represented by Area 5.

The most marked and characteristic clinical effect of uncompensated alkali excess is the development of symptoms of tetany when the alkalinization proceeds sufficiently far. From this fact, however, one is not at present justified in assuming that all tetany is either caused by, or accompanied by, alkalosis. MacCallum and his coworkers did not find high plasma bicarbonate in tetany caused by parathyroidectomy, although McCann (1918) did find it.

Areas 2 and 3.—Uncompensated CO_2 Deficit.—In this condition the $[H_2CO_3]$ is decreased without a parallel fall in $[BHCO_3]$. The result is, therefore, as in the condition represented by Area 1, an increase in the $[BHCO_3]$: $[H_2CO_3]$ ratio and the pH, but it is due to loss of $[H_2CO_3]$ instead of increase in $[BHCO_3]$.

Area 2 represents the first result of lowering in blood $[H_2CO_3]$ by a respiratory stimulus other than either the blood hydrion or H_2CO_3 concentration. The consequence is an overalkaline reaction.

A compensating retention of acid metabolites, indicated by a decreased excretion of ammonia and titratable acid in the urine

sets in, and there is also, as when the pH is raised by increased [$BHCO_3$], an excretion of bicarbonate (Davies, Haldane, and Kennaway). As a result of these compensatory processes the bicarbonate of the blood may be lowered in some hours from Area 2 to Area 3 (partial compensation), and eventually to Area 6, where the pH is again down to its normal value (entire compensation). This last condition is attained when one becomes acclimated to a high altitude (Hasselbalch and Lindhard, 1915).

Uncompensated CO_2 deficit has been caused in man by hyperpnea either voluntary (Collip and Backus, 1920; Davies, Haldane, and Kennaway, 1920; Grant and Goldman, 1920; Milroy, 1914), or induced by breathing air with a diminished oxygen content, such as is encountered at high altitudes (Haggard and Henderson, 1920; Haldane, Kellas, and Kennaway, 1919). Bazett and Haldane (1921) have observed it as the result of hyperpnea caused by emersion in warm water. Apparently unusual demands on the lungs for either oxygenation or cooling may arouse respiratory stimuli which to some extent rob the hydrion stimulus of its usual control.

The effects of uncompensated CO_2 deficit on the urine are similar qualitatively to those of uncompensated alkali excess; there is a decrease in ammonia excretion, an increase in urinary pH, and an excretion of bicarbonate (Davies, Haldane, and Kennaway). The rate of bicarbonate excretion observed, however, was much less (only a fraction of a gram per hour) when the blood pH was raised by overbreathing than when it was raised by administration of bicarbonate.

The ultimate clinical symptoms are again those of tetany and have been identified as such by Grant and Goldman (1920). The characteristic signs after voluntary deep breathing for an hour or less included the carpopedal spasm, Chvostek's sign, Trousseau's sign, Erb's sign, and in one instance even a tetanic convulsion. The physiological effects of abnormally high blood pH appear to be similar, whether the increase is caused by an increase in the numerator or a fall in the denominator of the [$BHCO_3$] : [H_2CO_3] ratio.

Area 4—Compensated { *Alkali Excess or* / *CO_2 Excess.* } —Here the pH is normal, the [$BHCO_3$] is high but is balanced by a proportionally high [H_2CO_3]. The state of the acid-base balance of the blood is the same, whether

the original disturbance is alkali retention (Area 1) or CO_2 retention (Areas 7 and 8). Hence the condition may be described as either compensated alkali excess or compensated CO_2 excess, according to whether the primary disturbance is due to alkali or CO_2 retention. The condition indicated by Area 4 has been observed to arise from both causes.

Alkali excess has been observed after therapeutic overadministration of sodium bicarbonate. If, as is usually the case following moderate oral administration, the absorption is not rapid, CO_2 may be retained sufficiently to balance the increased [$BHCO_3$], and the condition changed from that indicated by Area 5 merely to that of Area 4. If absorption is too rapid for simultaneous compensation by CO_2 retention, the condition changes to that indicated by Area 1, presumably to return later through Area 4 to normal Area 5.

Compensated CO_2 excess appears to be the state observed by Scott (1920) in emphysema. The retarded gas exchange presumably leads to a state of chronically increased CO_2 tension in the blood, and the body raises the blood [$BHCO_3$] high enough to balance the [H_2CO_3] and maintain a normal reaction.

It appears that this condition, primarily due to CO_2 retention, may be differentiated from that in which alkali retention is the primary cause, by the fact that the former is associated with cyanosis (as in emphysema), either permanent or caused by slight exertion. Diffusion of oxygen is so much slower than that of CO_2 (Krogh, 1919) that any hindrance retarding the alveolar gas exchange sufficiently to affect CO_2 excretion would presumably be accompanied by still more hindrance to oxygenation of the blood. This presumption is further supported by the work of Krogh and Krogh (1910), who found in rabbits that while CO_2 tension in arterial blood and alveolar air are equal, oxygen tension is lower in the blood than in the alveolar air, even when respiration is unhindered.

Area 5—Normal Acid-Base Balance.—The normal area represents the balance that is practically always found in the resting individual in health and at ordinary altitudes. (At higher altitudes the normal dissociation curve falls parallel with the barometric pressure (Y. Henderson, 1920)). Area 5 covers approximately the conditions represented in detail by the nomogram of L. J. Henderson (1921).

The minimum and the maximum normal arterial CO_2 tensions and bicarbonate concentrations indicated by the lower left and upper right corners of Area 5 are about twice as far from the means as are the normal extremes for these values heretofore estimated from alveolar air and arterial blood analyses. The wide range indicated by the diagram may be due to the fact that technical errors have widened in all directions the ranges indicated by the boundaries of Area 5. More accurate data will perhaps show this area to be smaller, and the extremes therefore less far apart. For the pH limits of the plasma Parsons is inclined to place the normal range at more nearly between pH 7.30 and 7.40 than the doubly wide range of 7.30 to 7.50 which we have allowed.

Another reason in part perhaps responsible for the fact that the extreme $[BHCO_3]$ and CO_2 tension values of Area 5 exceed the normally observed extremes is that there would be only one chance out of many for maximum pH and minimum $[BHCO_3]$, or the reverse, to occur in the same individual; *e.g.*, if levels of each so far from the mean are taken as to include only 1 individual out of 20, presumably only 1 out of 400 would show both extremes at once. Consequently it would not be surprising if the extreme normal limits of CO_2 tension and $[BHCO_3]$ have hitherto escaped observation, or have been observed so rarely as not to be regarded normal.

With more accurate technique and a larger number of observations it appears probable that the limits of normal arterial CO_2 tension and bicarbonate concentration indicated by a graphic estimation like the above will coincide with those observed.

Area 6.—Compensated { *Alkali Deficit or* / *CO_2 Deficit.* —Area 6 represents a condition in which the available blood alkali is lowered, but in which a normal pH is maintained because the fall in $[BHCO_3]$ is balanced by a proportional fall in $[H_2CO_3]$. The primary cause of the condition may be a fall in either $[H_2CO_3]$ or $[BHCO_3]$, decrease in the other factor being in each case a secondary balancing or compensatory process, with the apparent physiological object of maintaining a normal pH. Although the final state of the acid-base balance in the blood is in each case the same, *viz.* proportionally lowered $[BHCO_3]$ and $[H_2CO_3]$ with normal pH, the state may nevertheless with some

advantage be differentiated by two terms, as either compensated alkali deficit or compensated CO_2 deficit, according to whether the primary cause of the abnormality is deficit in the available alkali, caused by retention or overrapid formation of non-volatile acids, or whether it is deficit in $[H_2CO_3]$ caused by some respiratory stimulus added to or intensifying the usual $[H^+]$ stimulus. In alkali deficit the result of a failure in the secondary compensating processes would be acidification, in CO_2 deficit it would be alkalinization. Presumably different means, depending on the nature of the primary cause, may be required in each case to restore and maintain normality.[1]

Compensated alkali deficit is the condition occurring as the result of accelerated production of non-volatile acids (diabetes) or their retarded elimination (presumably the case in nephritis). The bicarbonate reserve of the entire body is diminished, that of the blood falling parallel with that of the other body fluids. In experimental intoxication of rabbits with HCl, Goto (1918) has found that the potassium phosphate of the tissues and the $CaCO_3$ of the bones are also reduced.

In the evident attempt to maintain a normal $[H_2CO_3]$: $[BHCO_3]$ ratio and pH in the blood, ventilation becomes deeper, and the $[H_2CO_3]$ is reduced in proportion to the $[BHCO_3]$. Presumably there is during the acid invasion a slight increase in hydrion concentration, causing the blood condition to shift toward the border which separates Areas 6 and 9, The respiratory center is, however, at once stimulated, and CO_2 is driven off so that until compensation breaks down pH is kept within normal limits and the condition remains in Area 6. At the same time accelerated formation of ammonia and excretion of buffer acids, such as acid phosphate, tend to raise the available alkali back to normal.

Compensated alkali deficit is the condition commonly observed as the result of retention of non-volatile acids in metabolic diseases, such as diabetes and nephritis (discussed by Van Slyke and Cullen, 1917) and marasmus of infants (Schloss and Harrington, 1919; Howland and Marriott, 1916). Until recently it has been the only

[1] Whether the lowered blood alkali of traumatic shock is primarily due to non-volatile acid retention (Cannon, 1918) or to CO_2 loss (Y. Henderson and Haggard, 1918) is still uncertain.

form of naturally occurring acidosis recognized clinically, except the uncompensated acidosis (Area 9) of the premortal state. Since the pH is normal, the alkali neutralized by the invading acids is solely that of the bicarbonate (see page 17), and the fall in blood bicarbonate is an exact measure of the non-volatile acid that enters the blood.

For the reason that this condition at the time represented all clinically observed acidoses except the uncompensated premortal acidosis, in which also, however, the bicarbonate is reduced, Van Slyke and Cullen in 1917 defined acidosis as a condition in which the blood bicarbonate is lowered. The definition is adequate for the forms of acidosis caused by retention of non-volatile acids, but it does not cover the conditions since observed which are represented by Areas 7 and 8, and which are caused by CO_2 retention; and it fails to exclude the conditions represented by Areas 2 and 3, in which the bicarbonate is reduced as a result not of acid retention but of CO_2 loss. With apparent adequacy, however, one may define acidosis as a condition caused by acid retention sufficient to lower either the bicarbonate or the pH of the blood below the normal limits.

Compensated CO_2 Deficit—As already stated above, a fall of blood alkali with maintenance of normal pH may also occur when the primary cause is not acid retention, but excessive respiratory loss of CO_2. In this case the fall in blood bicarbonate is a compensatory process which tends to prevent the blood reaction from becoming abnormally alkaline. One respiratory stimulant which has been demonstrated to have such an effect is oxygen want.

Y. Henderson (1920) has shown from data obtained by Fitzgerald, and by Douglas, Haldane, Henderson, and Schneider (1912) on their Pike's Peak expedition that the CO_2 of the alveolar air is lowered at high altitudes, and varies in direct proportion to the barometric pressure. This is also shown by the data of Hasselbalch and Lindhard (1915). Since the rate of CO_2 production is not lowered (Hassebalch and Lindhard), it is evident that the minute volume of air breathed is increased, an effect which is also noted when air with reduced oxygen percentage is breathed at sea-level pressure.

The process by which the state of compensated CO_2 deficit is reached has been outlined in the discussion of the condition represented by Areas 2 and 3.

The final effect, lowered bicarbonate with normal pH, is the same as in compensated retention of non-volatile acids. The primary cause, however, is not acid retention, but loss of an acid (carbonic) which is compensated by a reduction of the blood alkali.

Areas 7 and 8—Uncompensated CO_2 Excess.—In this condition respiratory excretion of CO_2 is retarded, either by physical hindrance or by deadening of the respiratory center, so that the $[H_2CO_3]$ of the blood is raised. In consequence the $[BHCO_3] : [H_2CO_3]$ ratio and the pH are lowered. The actual blood reaction becomes less alkaline than normal.

This condition has been caused experimentally by breathing air which contains 3 to 5 per cent of CO_2 (Hasselbalch and Lundsgaard, 1912; Davies, Haldane, and Kennaway, 1920). It appears to be caused also when the respiratory center is deadened by morphine narcosis (Michaelis and Davidoff, 1912; Henderson and Haggard, 1918).

Means, Bock, and Woodwell (1921) report an observation of the condition in a cyanotic pneumonia patient.

The physiological effects are seen in an accelerated excretion of ammonia and titratable acid by the urine, the same as when the acid-base balance is shifted towards the acid side by retention of non-volatile acids. Davies, Haldane, and Kennaway (1920) observed a doubling of the rate of ammonia and titratable acid excretion after breathing air containing up to 5 per cent of CO_2. There is also, as in non-volatile acid retention, an increase in the minute volume of air expired in the apparent attempt to get rid of the excess of CO_2, unless the respiratory center is deadened, as by morphine.

The first effect of CO_2 retention on the blood is to increase the $[H_2CO_3]$ and $[H^+]$ of the blood, without changing the buffer alkali content (condition represented by Area 8). Thus Davies, Haldane, and Kennaway found after breathing for an hour air containing CO_2 in amounts gradually increasing up to 5 per cent, there was no change in the CO_2 capacity of the blood (unchanged total buffer alkali). Henderson and Haggard (1918) found in dogs a rise of 2 or

more volumes per cent in CO_2 capacity of the blood within a half hour after injecting morphine, or breathing air containing 5 or more per cent of CO_2.

The increase in the blood alkali occurring in Area 7 is secondary to the $[H_2CO_3]$ increase and is compensatory in its nature; it tends to raise the $[BHCO_3] : [H_2CO_3]$ ratio back to normal by increasing the $[BHCO_3]$ to balance the increased $[H_2CO_3]$. In consequence the blood condition from Area 8 shifts to Area 7 (partial compensation). Normal Area 5 is presumably regained either by passing directly back to Area 5 from Area 8 (in case the excess CO_2 is blown off before secondary rise of alkali to Area 7 occurs), or by passing through Areas 7 and 4 to 5 (in case compensation is accomplished partly by the secondary rise in alkali).

The compensatory increment of blood alkali in Area 7 probably comes from two sources: (1) The increased excretion of ammonia and titratable acid through the kidneys tends to raise the bicarbonate content of the entire body, and the blood plasma bicarbonate would normally rise with that of the other fluids. (2) HCl may perhaps leave the blood plasma and enter the tissue cells, as it has been shown to leave the plasma and enter the blood cells when the pH rises (for discussion and literature on this electrolyte shift see Van Slyke, 1921). The rate of blood alkali rise observed by Henderson and Haggard appears too rapid to be probably accounted for by acid excretion alone, and these authors attribute the increase to alkali drawn from the tissues. The effect would be the same if acid passes from the blood into the tissues, a process which from analogy with the shift between plasma and blood cells seems more probable. The relative parts that these two factors, acid excretion and shift of acid to the tissues (or of alkali in the reverse direction), play in the compensatory rise of blood bicarbonate during CO_2 retention is uncertain. That accelerated acid excretion occurs has been shown. That acid shift from blood to tissues also occurs seems probable.

Area 9.—Uncompensated Alkali Deficit.—In this condition the $[BHCO_3]$ of the blood is lowered without a proportional fall in $[H_2CO_3]$. In consequence there is a fall in the $[BHCO_3]:[H_2CO_3]$ ratio and the pH.

This is the condition defined by Hasselbalch and Gammeltoft (1915) as "uncompensated acidosis." It has been most frequently

observed in cases of nephritic and diabetic acidosis in the premortal period. Means, Bock, and Woodwell (1921) describe both the symptoms and blood changes in such a nephritic case. The blood bicarbonate is extremely low. Respiration, which up to the terminal stage has kept the $[H_2CO_3]$ sufficiently low to maintain a normal $[H_2CO_3]:[BHCO_3]$ ratio, now fails to do so, and the blood condition shifts from that represented by Area 6 over into that represented by Area 9.

In deep ether anesthesia, according to the results of Van Slyke, Austin, and Cullen (1919-20), the blood state is represented by Area 9, and both alkali deficit and carbonic acid retention occur. This combination appears also to occur in some cardiac cases (Peters and Barr, 1921).

Relation of Changes in the Acid-Base Balance of the Blood to Changes in the Other Body Fluids.

As has already been shown, the intercellular fluids other than blood plasma have, so far as studied, been found under normal conditions to approximate the blood plasma in bicarbonate and hydrion concentrations. There is evidence that in changes from the normal the other body fluids follow more or less promptly the blood plasma. Van Slyke and Cullen (1917) found that when acid was injected into the circulation the fall in blood bicarbonate was only about one-sixth as great as it would have been had the acid all remained in the blood; the other five-sixths of the acid must have gone into the other body fluids and the tissues, or drawn alkali from them. Palmer and Van Slyke (1917) found similarly that when bicarbonate was administered the rise in blood bicarbonate was approximately that calculated on the assumption that the alkali was not retained in the blood, but was distributed evenly through all the body fluids. Collip and Backus (1920) found that the bicarbonate of the spinal fluid follows that of the venous blood plasma. When the latter was lowered by continued etherization, or by shock (handling of intestines), the bicarbonate of the spinal fluid also fell and to about the same level, although more slowly. When the bicarbonate of the blood was raised by bicarbonate injection, the spinal fluid bicarbonate rose in the course of a few hours to approximately the same level.

The Physiologically Available Alkali of the Bicarbonate and of the Other Blood Buffers.

In order that a buffer shall neutralize an acid (HCl for example) *without change in pH* it is necessary that the buffer acid HA, set free by the reaction BA + HCl = BCl + HA, shall be completely removed, and in addition as much of the HA formerly present as may be necessary to keep the ratio [BA]: [HA] at the original value. Of the blood's important buffers, plasma protein, cell phosphates, hemoglobin, and bicarbonate, only the bicarbonate has an acid which can be quickly removed. Under nearly all circumstances, physiological and pathological, in which the respiratory apparatus is not specifically affected, it appears that the $[H_2CO_3]$ is so regulated that a normal pH is maintained. This is accomplished even by the ill diabetic or nephritic so efficiently that, until the $[BHCO_3]$ has been reduced by invading acids to one-fourth, and perhaps even one-eighth of its normal value, the $[H_2CO_3]$ is reduced in the same proportion, and a normal pH is maintained.

So long as the pH is kept constant in the above manner, all the changes in buffer alkali are those of the bicarbonate. For at a constant pH the ratio [BA]: [HA] remains constant for each buffer, in accordance with the general equation for salts of weak acids; *viz.*, $\frac{[HA]}{[BA]} = K_1 [H^+]$. Consequently, however much depletion the $[BHCO_3]$ may suffer, the alkali salts of the other buffers are unaffected.

However, when a fall in pH occurs, the alkali of the other buffers is drawn upon and, as mentioned before, conditions have been recently observed in which a fall in pH does occur. From the data available it appears that for a short time at least the pH may fall as low as 7.0, although not much lower without fatal results.

The maximum available alkali of the blood is therefore almost the entire alkali of the bicarbonate plus that portion of the other buffer alkalies which is yielded when the pH changes from normal to the minimum compatible with life. The amount of available buffer alkali may be estimated by increasing the CO_2 tension of the blood until the pH is reduced from 7.4 to 7.0. All the alkali given up by the other buffers is bound by H_2CO_3 and thereby turned into bicarbo-

nate under these conditions, so that the increase in bicarbonate above that at normal CO_2 tension and pH 7.4 represents the available alkali of the other buffers. By extrapolation of the average normal CO_2 absorption curve of human blood in Fig. 2 we find that this increase covers the range from 20.5 to 28.0 millimolecular [$BHCO_3$]. The available alkali from buffers other than bicarbonate is therefore 7.5 millimolecular in concentration, equivalent to $2.24 \times 7.5 = 17$ volumes per cent of CO_2, or approximately one-third the normal blood bicarbonate alkali.

The average total alkali of normal human blood available for neutralizing invading acids may therefore be summarized as:

0.0205 M bicarbonate alkali, (equivalent to 46 volumes per cent of bicarbonate CO_2). Of this three-fourths, and perhaps seven-eighths, may be used for neutralization of acid without change in pH and most of the remainder becomes available if the pH falls to 7.0.

0.0075 M alkali, (equivalent to 17 volumes per cent of bicarbonate CO_2), from other buffers, available only when the pH falls to 7.0.

Total 0.280 M available alkali, (equivalent to 63 volumes per cent of bicarbonate CO_2).

Of the 0.0075 M alkali available from buffers other than bicarbonate the greater part is normally bound to hemoglobin (Van Slyke, 1921, p. 160).[2]

In a broad sense, therefore, the alkali reserve of the blood includes not only the bicarbonate, but in addition about one-third as much alkali from the other blood buffers. In a still broader sense one might add the alkali of the bicarbonate and other buffers in the tissues and body fluids outside the circulation, since it also becomes

[2] If the amounts of alkali bound to oxyhemoglobin at pH 7.4 and 7.0 are calculated by the equation $\frac{[BHCO_3]}{[HHCO_3]} = pH - pK$, placing $[BHCO_3 + HHCO_3]$ equal to 0.045 M and pK, for hemoglobin equal to 7.2 (Van Slyke, 1921), we calculate in fact that the oxyhemoglobin in normal blood would yield 0.010 M alkali in changing from pH 7.4 to 7.0. This is $\frac{1}{3}$ more than all the buffers together yield according to the curves of Fig. 2. The quantitative discrepancy will presumably disappear when the constants for hemoglobin are more accurately determined, and perhaps also when the normal CO_2 absorption curves in the pH range 7.3 to 7.0 are more accurately worked out.

available when the blood is flooded with acid (Van Slyke and Cullen, 1917, p. 338). In the sense, however, that it contains the only alkali that can neutralize acids without fall in blood pH, the bicarbonate forms a reserve in a class by itself.

Means for Determining State of the Acid-Base Balance.—It is not the purpose of the present paper to discuss in detail the technique for studying the acid-base balance of the blood and the body, but the principles appear derivable from the preceding discussion.

In order to determine which one of the possible variations exists in the blood *in vivo* it is necessary to ascertain two of the involved variables, such as the pH, $[BHCO_3]$, and $[H_2CO_3]$. With any two of them a point can be located in its proper area on a diagram such as Fig. 1, 2, or 3, but with any one of them alone it cannot be done.[3]

Under most conditions, pathological as well as normal, the pH is kept normal. When this is the case determination of either the CO_2 tension or the bicarbonate in either plasma or whole blood suffices to indicate the condition. The plasma bicarbonate determination by the gasometric method of Van Slyke and Cullen (1917) or the titration method of Van Slyke, Stillman, and Cullen (1919) is adequate for this case, and therefore suffices for the study of metabolic conditions (*e.g.* those usually met in diabetes, nephritis, and metabolic disturbances of infants) in which the source of acid-base disturbance is retention of non-volatile acids, while the respiratory control of the blood reaction is unaffected. It is not adequate to cover conditions in which the respiratory control of the blood reaction is so disturbed that the pH becomes abnormal, as happens in anesthesia.

Joffe and Poulton (1920) and Peters and Barr (1921) have suggested, as the preferable single blood determination, the CO_2 content of the

[3] When the blood is only partially saturated with oxygen, accurate location of its point on a diagram such as Fig. 1, based on figures from complete oxygenated blood, will involve a correction for the oxygen unsaturation. (The effect of oxygenation and reduction of hemoglobin in the blood bicarbonate was discovered by Christiansen, Douglas, and Haldane (1914) and has been discussed theoretically by L. J. Henderson (1920) and by the writer (1921).) For a diagram such as Fig. 1, with CO_2 contents as ordinates and CO_2 tensions as abcissæ, Peters and Barr (1921) have estimated the approximate correction as −0.34 volume per cent of CO_2 for each volume per cent of oxygen unsaturation.

whole blood determined after equilibration with air containing CO_2 at 40 mm. tension. This estimation is sufficient to indicate whether the available alkali is normal or abnormal, but to indicate the entire state of the acid-base balance it is inadequate. Thus from reference to Fig. 1 it is evident that a CO_2 capacity of 30 volumes per cent determined at 40 mm. CO_2 tension on whole blood indicates a bicarbonate reserve about 12 volumes per cent (in CO_2 terms) below the minimum normal. Whether the condition existing in the body is that indicated by Area 3, 6, or 9, is, however, left uncertain.

In regard to the use of such diagrams as have been employed in this paper a conclusion indicated by results of Hasselbalch, of Parsons, and of Peters and Barr may be reiterated; *viz.*, that in order to draw the most accurate deductions concerning the variations in the acid-base balance of a given individual it may be necessary to know the conditions that are normal for his particular blood. For each individual Area 5 of our diagram presumably shrinks to a fraction of the area, indicated on the charts in this paper, required to include the normal variations of the species.

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THE FOOD REQUIREMENTS OF CHILDREN.

II. Protein Requirement.*

By L. EMMETT HOLT, M.D., and HELEN L. FALES.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital, New York.)

In the first paper of the series on the food requirements of children the total caloric need throughout the entire period of growth was discussed in detail. The next step in the consideration of food needs is a study of the distribution of the total calories necessary. This involves the estimation of the required amounts of fat, carbohydrate and protein to be supplied in the daily diet and the relative proportions of each of these. When the total caloric need is known the proportion of the different constituents is most concisely expressed by the percentage of fat, carbohydrate and protein which are given.

All three of these nutritive substances are sources of energy. There are, however, other important considerations which affect the amount of each one of them in the diet. Especially is this true of the protein. Before considering the other food elements, it is necessary, first, to establish the protein requirement. Proteins differ greatly in their composition and it is impossible to consider the total protein requirement without taking these differences into account.

As is well known, the food proteins are composed of fifteen or twenty amino-acids which are combined in varying proportions in the different proteins. In the intestines, the food proteins are split up into amino-acids which are then absorbed and subsequently recombined to form the proteins of the body. The proportions of amino-acids in the body proteins are not the same as in the food proteins. As a consequence, not all the amino-acids of the food are utilized in building up or repairing the body structure. Those amino-acids which are not utilized as building materials are used largely for energy,

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being broken down into urea and excreted in the urine. A food protein may furnish a considerable excess of one or more of the amino-acids needed as building material, while at the same time it may be deficient in other necessary amino-acids. Certain of the amino-acids, such as leucin, glutamic acid, arginin, prolin, etc., which form large proportions of the body proteins, are present in abundance in all food proteins; consequently an ample supply of these is always assured. Other amino-acids which are especially needed in growth, such as lysin, cystin, tryptophan, etc., are found in much smaller and in widely varying proportions in the different food proteins. Many of the proteins are in fact entirely wanting in one or another of these amino-acids. Hence, special care must be taken to provide in the diet of growing children either the proteins having the larger proportion of these important amino-acids or else there must be furnished a greatly increased supply of the proteins which have a lower proportion of these substances.

It is obvious that the total protein requirement for children during the period of growth is to a great extent dependent on the character of the protein given. The animal proteins approximate more nearly the proteins of the human body than do those of vegetable origin and for this reason are preferable. For the growing child, therefore, the animal proteins, such as are furnished in milk, eggs and meat, are distinctly of a higher grade than the vegetable proteins. It is possible that when the knowledge of the structure of the body proteins has progressed further, a combination of vegetable proteins might be made to approximate for nutritive purposes the quality of animal protein. With our present knowledge, however, it appears necessary, if vegetable proteins are depended on, to supply them in large amounts, in order to insure normal growth.

In estimating the child's protein need two functions of protein must be considered. The first is the requirement for tissue repair and general maintenance; the second is that for growth. In adult life the first need is the only one to be supplied. In childhood the second is equally important. Even the protein requirement of the adult has been the subject of much controversy. It has been found possible to maintain nitrogen equilibrium in an adult man or woman on a protein intake as low as 40 gm. daily. Some writers believe that this

minimum allowance is a sufficient supply. The average adult dietary, however, as compiled from many sources, contains from 100 to 120 gm. protein daily. This naturally adjusted intake has been regarded by many as an expression of the optimum. This amount of protein allows an average of about 1.4 gm. protein per kilo of body weight.

Sherman[1] recently found the average protein minimum in 109 adults to be 44.4 gm. daily. This represents 0.635 gm. per kilo for a person of average weight. He concludes that in an adult 1.0 gm. protein per kilo is an ample allowance for maintenance. The work of various investigators indicates that the entire need for maintenance can probably be supplied by vegetable protein. Sherman, however, believes that better results are obtained by supplying part of the maintenance requirement by animal protein. Among the vegetable proteins certain ones, especially zein—the protein of corn—are insufficient even for maintenance.

From the knowledge of the adult protein need it may reasonably be inferred that the growing child requires for maintenance from 1.0 to 1.5 gm. per kilo of body weight daily. It is probable that this can be supplied either from vegetable or animal sources. In addition to the maintenance need, however, the child must be supplied with a generous amount of protein for growth. Because the vegetable proteins as a group are so poor in certain of the important amino-acids it seems desirable, and probably necessary, to supply the growth need by animal protein. How much protein should be furnished to cover this requirement is difficult to estimate.

No experimental work has been reported on the protein need of children and the effect on their growth of either a very low or very generous protein intake. Osborne and Mendel[2] have been able to obtain quantitative results on protein needs in their work with rats. They have determined exactly how much of certain proteins will support normal growth in rats and also to what extent the amount of these proteins in the diet can be reduced if their quality is improved by the addition of certain amino acids in which they are known to be deficient. Such experimentation is by its very nature quite im-

1. Sherman, H. C.: J. Biol. Chem. **41**:97, 1920.

2. Osborne, T. B., and Mendel, L. F.: J. Biol. Chem. **25**:1, 1916, id. **37**:223, 1919, and others.

possible with children. Accordingly, about the only guide to a knowledge of the total protein need of children is found in a study of the actual amounts of protein taken by normal healthy children in their customary diet. We fully realize that the results of this method of investigation are not conclusive, but they are strongly suggestive. They, therefore, deserve careful consideration until further light is shed on the question by more extended observations, possibly different in character.

The protein intake of the normal nursing infant is very low. It ranges during the first nine months of life approximately from 8.0 to 12.0 gm daily. This is equivalent to only about 1.5 gm. per kilo. Up to the age of 8 or 9 months the protein taken by the nursing infant seldom exceeds 12 gm. daily. This amount must be sufficient to supply not only the maintenance need but the need for growth during the most active period of growth in the life of the child, for we find our best examples of good nutrition and growth in breast-fed infants. The reason for the adequacy of this small protein intake is that the protein of woman's milk is certainly best adapted in its amino-acid composition for digestion and assimilation by the infant.

When cow's milk is substituted for woman's milk experience has shown that the protein intake must be considerably increased—doubled or even trebled. Infants from 1 month to 9 months of age receive from 15 to 30 gm. protein daily when fed on the usual modifications of cow's milk. This represents fully 3.0 gm. and often 4.0 gm. protein per kilo. The increase in protein requirement when cow's milk is substituted for woman's milk is probably due to the difference between the two milks in amino-acid content. Woman's milk contains about twice as much lactalbumin as does cow's milk. The work of Mendel and Osborne, as well as that of others, has shown that lactalbumin is especially efficient in promoting growth, while casein, which forms five-sixths of the protein of cow's milk, is of comparatively low grade as a growth protein, being notably deficient in cystin. Furthermore, lactalbumin, which forms two-thirds of the protein of woman's milk, contains of all the proteins the highest proportions of the amino-acids leucin, lysin and tryptophan.

The failure of condensed milk as a food for infants has been ascribed to its low fat and its relatively high sugar content. It now seems

clear that equally important—probably more important than either of these—is its low protein. As usually fed in dilutions of one to eight or one to six, the protein ranges from 1.0 to 1.4 per cent., which is only about that of woman's milk.

Again, in the early days of milk modification by the percentage method it was thought that cow's milk mixtures in which the proportions of fat, sugar and protein were as nearly as possible those found in woman's milk would be the best substitute for it. The many failures from the use of such formulas have been attributed to the high fat, but it now seems quite evident that the low protein was even more at fault. It has become increasingly clear with clinical experience that the majority of infants will not thrive normally on cow's milk unless they are given two or three times as much protein as is contained in woman's milk.

The proportion of vegetable proteins in the diet of infants is a very small one. Whether nursed or artificially fed the infant receives practically his entire protein supply in the form of animal protein at least during the first six months of life. It is only when cereal additions are given, either as flour incorporated in the milk formula or as cereal given separately, that a portion, although at first a very small one, of the protein given is of vegetable origin.

Very little attention has thus far been paid to the quantitative composition of the diet of children over 1 year of age. There is, therefore, very little material in the literature concerning the actual daily amount of protein taken by older children. The few scattered data published are for the most part incidental to researches on other subjects. Most of the authors whose reports on the total calories taken by children were discussed in the first paper of our series have also reported the protein content of the diets taken.

Gillett[3] has compiled from eighteen authors 146 such cases, including most of the values for protein intake given in the literature up to 1917, and has summarized them in her article. This summary is shown in Table I. It will be seen that in all the groups the average intake was at least 2 gm. protein per kilo and that the younger children took on the average 3 gm. per kilo.

3. Gillett, Lucy H.: Publication 115, N. Y. Assn. for Imp. Cond. of Poor, 1917.

Müller,[4] whose cases are included in Gillett's summary, discusses in considerable detail the protein intake of the thirty-two children studied by him. These children, from 1 to 6 years of age, took on the average 3.5 gm. protein per kilo daily. It must be borne in mind, however, that these children were much underweight. Müller thinks that the protein need of children is proportionally very much greater than that of adults, and that no attempt should be made to reduce the protein intake to a minimum lest growth be interfered with and general nutrition impaired.

TABLE I.

Summary of Literature on Protein Intake Per Kilo (Gillett).

Age, Years	Number of Cases	Total Protein, Gm. Daily	Protein Intake, Average Gm. per K.
2– 5	51	53	3.3
6– 9	44	67	2.6
10–13	33	73	2.0
14–17	18	94	2.2

Total range in all cases over one year, 1.37–4.86.

TABLE II.

Average Protein Intake in Gephart's Observations.

	Average Age: Yrs.	Mos.	Total Protein, Gm. Daily	Protein Intake, Average Gm. per K.
Lower school	13	6	164	3.76
Middle school	14	7	165	3.26
Upper school	16	1	157	2.60

Gephart[5] in his observations on the adolescent boys in St. Paul's School found the protein intake to be very high. The average total daily protein was about 160 gm.; while the average protein per kilo ranged from 2.6 to 3.8 gm. per kilo. His averages are shown in Table II.

Camerer,[6] whose schedule for feeding older children has been widely followed, recommends over 4 gm. per kilo for the first year, from

4. Müller, E.: Biochem. Ztschr. 5:143, 1907.
5. Gephart, F. C.: Boston M. & S. J. 176:17, 1917.
6. Camerer: Stoffwechsel des Kindes, 1896.

TABLE III.

Average Protein Intake of the Children Studied.

Age, Years	Boys					Girls					Both Sexes				
	Number of Cases	Total Protein Gm. Daily	Protein Gm. per K.	Per Cent Animal Protein	Per Cent Vegetable Protein	Number of Cases	Total Protein Gm. Daily	Protein Gm. per K.	Per Cent Animal Protein	Per Cent Vegetable Protein	Number of Cases	Total Protein Gm. Daily	Protein Gm. Per K.	Per Cent Animal Protein	Per Cent Vegetable Protein
1–2	2	32	3.2	75	25	5	49	4.4	70	30	7	44	4.1	71	29
2–3	4	48	3.4	71	29	7	56	4.1	72	28	11	53	3.9	71	29
3–4	6	51	3.8	71	29	4	68	4.4	68	32	10	58	4.0	70	30
4–5	7	57	3.3	70	30	5	53	3.0	70	30	12	56	3.2	70	30
5–6	5	63	3.3	70	30	5	58	3.3	64	36	10	60	3.3	67	33
6–7	4	70	3.0	63	37	4	50	2.5	68	32	8	60	2.7	67	33
7–8	2	69	2.7	56	44	3	66	2.7	59	41	5	67	2.6	58	42
8–9	4	80	3.1	71	29	5	75	2.7	70	30	9	78	2.9	70	30
9–10	5	88	3.0	63	37	4	68	2.5	68	32	9	80	2.8	64	36
10–11	6	107	3.5	69	31	3	73	2.2	64	36	9	96	3.1	68	32
11–12	3	82	2.2	64	36	1	77	2.1	73	27	4	80	2.2	64	36
12–13	1	87	1.9	59	41	2	73	1.6	69	31	3	78	1.7	65	35
13–14	..	...	...	..	..	1	74	1.7	60	40	1	74	1.7	60	40
14–15	2	162	3.4	63	37	1	66	1.4	57	43	3	130	2.4	63	37
15–16	2	134	2.4	67	33	1	111	2.1	64	36	3	130	2.3	65	35
16–17	1	116	2.2	56	45	..	...	...	..	..	1	116	2.2	56	45
17–18	..	...	...	..	..	1	115	2.0	82	18	1	115	2.0	82	18

3 to 4 up to the eighth year, over 2 gm. until the fourteenth year, diminishing to 1.7 gm. at the end of the growth period.

In a former article we[7] presented the values for total calories taken by over one hundred healthy children from one to seventeen years of age. In each of these cases we obtained complete diet records for a period of four days. These children were from intelligent well-to-do families and may be assumed to be taking diets which are fairly representative of what children under such circumstances usually receive. A study of the amount of protein taken by these

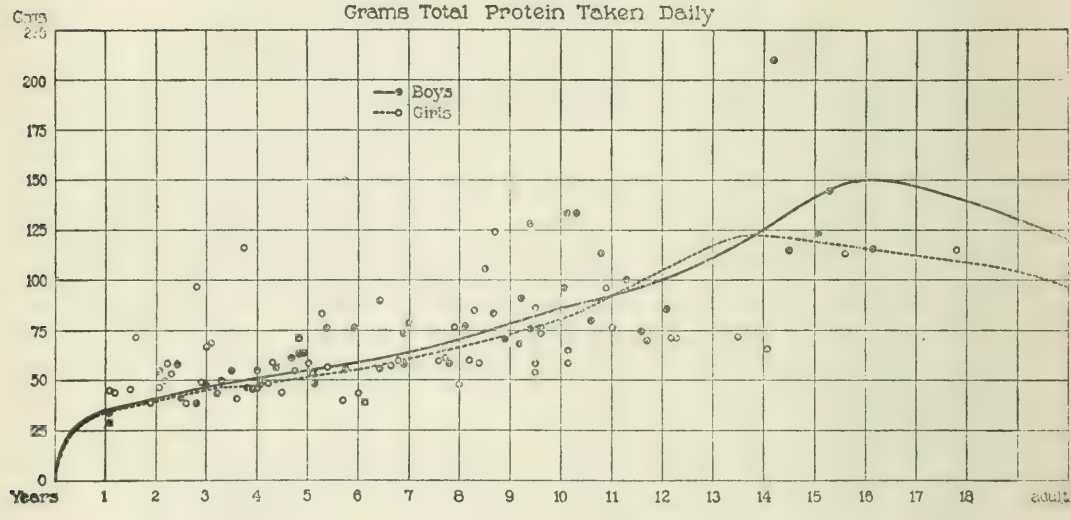

FIG. 1.

children should therefore, give some idea of the usual amount in the diet of healthy children. The average values for the total protein, the protein per kilo and the proportion of animal and vegetable protein taken at different ages are given in Table III. The average total protein in the second year was 44 gm., in the sixth and seventh year 60 gm., in the twelfth year 80 gm., in the fourteenth to the sixteenth year 130 gm. and in the eighteenth 115 gm.

Figure 1 shows the total daily intake of protein of the individual children. Our observations indicate that approximately 15 per cent.

7. Holt, L. E., and Fales, H. L.: Am. J. Dis. Child. 21:1, 1921.

of the total calories taken by children over 1 year old should be supplied by protein. The solid and dotted lines represent for boys and girls the grams of protein which are equivalent to 15 per cent. of the calories recommended in our previous paper for the different ages. While the great majority of these cases fall reasonably near the average lines there are some notable exceptions.

There are four girls whose protein intake was very high; three were between 18 months and 4 years of age and one was $8\frac{1}{2}$ years old. All four were extremely active children in excellent health whose total food intake was very high. Two large girls of $13\frac{1}{2}$ and 14 years,

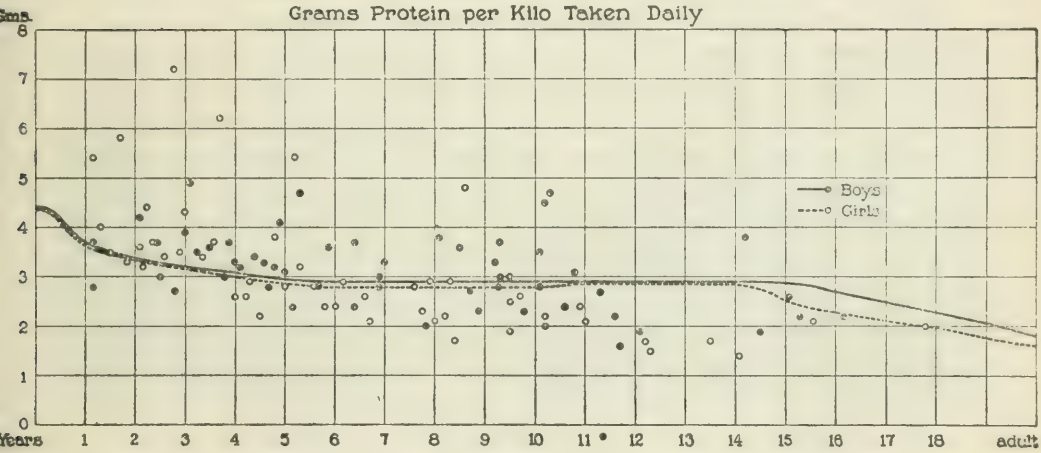

FIG. 2.

respectively, took a very low amount of protein. Both had attained practically their full growth, and for them really the only protein need was that for maintenance.

There were three boys about 10 years old with very high intake. The explanation in each case was excessive activity. A 14 year old boy taking 210 gm. protein daily is an example of what is so often observed, that is, the very large amount of food frequently taken by an active rapidly-growing boy at this age.

The grams of protein per kilo of body weight taken by these children are shown in Figure 2, the dots indicating the values for boys, the circles those for girls. The solid and dotted lines represent the

values for grams of protein per kilo equivalent to 15 per cent. of the calories per kilo recommended for different ages in our previous paper. Four-fifths of all the children observed took between 2 and 4 gm. protein per kilo. Only nine took less than 2 gm. per kilo, all of these children being over 8 years of age. Fourteen took more than 4 gm. per kilo. These were mostly young and very active children, only four being over 5 years of age. In general, the amount taken by the younger children was largest, the intake per kilo tending to diminish as age advanced.

It is seen that the protein intake of the children under 6 years averaged over 3 gm. per kilo. That of the older children averaged over 2 gm. per kilo for most years, there being a tendency to a reduction with the years. The values for girls between 12 and 15 are low, but there are too few observations to warrant deductions. It is probable that with more data the average protein intake of these years would be higher than is shown in Table III since these are the years of most rapid growth, and as we have shown in the discussion of total caloric needs, the food requirements are very high during adolescence.

These observations on normal children have also been analyzed with respect to the proportion of animal and vegetable protein in the diets taken. Most of the children took over 60 per cent. of their total protein in the form of animal protein, from milk, eggs, meat, etc. The average of all the cases shows that 66 per cent. of the total protein was animal and 34 per cent vegetable protein. In only one instance did a child take less than half of his protein as animal protein. Figure 3 shows graphically the percentage division of the total protein in the diet of each child. The values are arranged in order of age.

That there was found so little variation in the proportion of animal and vegetable protein in the different cases was to us a matter of much surprise. There are only three values for animal protein below 55 per cent. and only four above 80 per cent. of the total protein. Figure 3 shows clearly that there was no constant variation according to age. The older children received practically the same proportion of animal and vegetable protein as did the younger ones.

From the foregoing observations it is seen that healthy children take in their usual diet about 4 gm. protein per kilo at the age of 1 year, the amount diminishing to about 2.6 gm. at 6 years of age and

remaining at about this value or slightly below it until the end of growth. Of this total practically two-thirds is animal protein. Thus, there is in the usual diet about 2.7 gm. animal protein and 1.3 gm. vegetable protein at the age of 1 year and about 1.6 gm. animal protein and 1 gm. vegetable protein at the age of 6 years and thereafter. This quantity of protein may, perhaps, be regarded as the optimum and may, therefore, be recommended as a suitable intake for the growing child.

Recent investigations indicate that a low protein intake, or a protein intake of poor quality, as when that of the diet is largely of vegetable origin, is an important factor in the production of pellagra. This

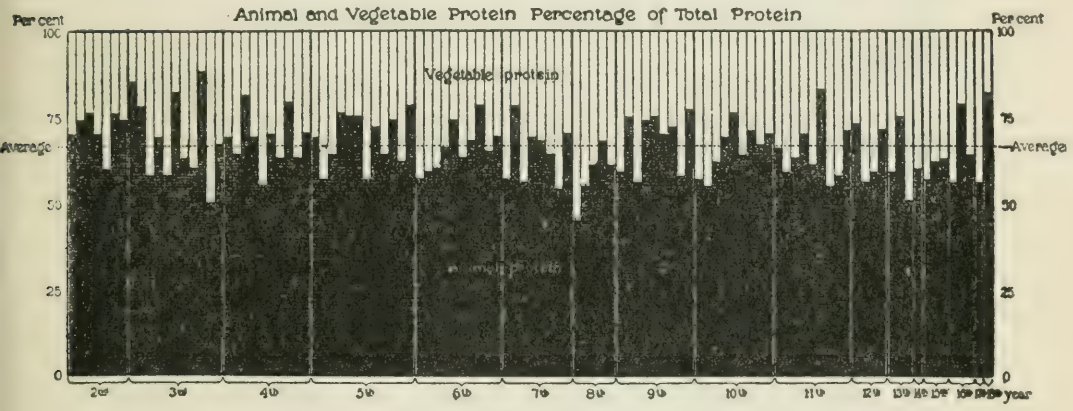

FIG. 3.

furnishes another argument in favor of a generous protein intake for growing children, and emphasizes the importance of providing the greater part of the protein from animal sources.

It is quite possible that children might do well if a smaller proportion of the protein given were animal protein. However, from what is known of the growth properties of the vegetable proteins as a class it is evident that a much larger amount of vegetable protein would be required to furnish the amino-acids necessary for normal growth.

Vegetable protein cannot replace animal protein gram for gram but must be given in greater amounts. Accordingly, if it is desired

to give a larger proportion of vegetable protein than the usual one-third of the total requirement the total protein intake must be considerably increased. When, however, the amount of vegetable protein given is increased very much above the average—that is, about 1 gm. per kilo—the amount of carbohydrate which must be necessarily taken with it, because of the nature of the occurrence of the vegetable protein, is likely to be excessive and may be a menace to proper digestion.

SUMMARY.

1. No estimate of the amount of protein required by growing children can be made without taking into account the kind of protein given, since the amino-acid content of different proteins varies considerably.

2. In estimating the protein needs of the child not only must the need for maintenance be supplied, as with the adult, but also a liberal amount of protein must be allowed for growth.

3. Vegetable proteins as a class are of a distinctly lower grade than animal proteins. While they may be adequate for maintenance, with our present knowledge it is hazardous to depend on them for growth.

4. The fact that the small amount of protein taken by nursing infants is adequate for healthy growth is due to the superior amino-acid composition of the proteins of woman's milk.

5. When cow's milk is substituted for woman's milk the amount of protein allowed should be doubled or trebled.

6. The average amount of protein taken in the usual mixed diet by over one hundred healthy children ranged from 44 gm. daily in the second year to 130 gm. daily in the fifteenth year. The latter figure exceeds the average adult need and obviously should do so, since adults require protein for maintenance only.

7. The amount of protein per kilo of body weight taken by these children averaged about 4 gm. at one year, diminished to about 2.6 gm. at 6 years and remained at about this value until the end of growth.

8. The children studied by us took about two-thirds of their protein as animal protein and one-third as vegetable protein.

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STUDIES ON BACTERIAL NUTRITION.

III. Plant Tissue, as a Source of Growth Accessory Substances, in the Cultivation of Bacillus influenzæ.

By THEODOR THJÖTTA, M.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 1, 1921.)

The preceding papers (1–3) in this series have dealt with the nature and action of the substances contained in blood which are of importance in the growth of *Bacillus influenzæ*.

Search for substances other than the hemoglobin of warm blooded animals, which might be capable of stimulating growth of *B. influenzæ*, has been made by several investigators. Davis (4) found that substances similar in their function to hemoglobin, namely hemocyanin, hemoerythrin echinochrom, did not sustain growth. He was unable to find any growth-inducing action in substances which readily give up oxygen, such as hydrogen peroxide and colloidal platinum, and among the various salts and organic preparations of iron he found none capable of replacing hemoglobin. Davis (5) has confirmed the observation of Ghon and von Preyss (6) that *B. influenzæ* will grow on hematin agar only in the presence of dead or living bacteria. Davis points out that while hemoglobin in plain agar yields growth, still more abundant multiplication occurs when hemoglobin is associated with bacteria or tissues, either plant or animal, and especially if these are living. However, he states that plant and animal tissues, and bacteria by themselves will not sustain growth.

Olsen (7), studying the effect of blood on growth of Pfeiffer's bacillus, tested the growth-inducing action of various constituents of blood and derivatives of hemoglobin. He found that serum and ether extracts of washed blood corpuscles were ineffective. He observed, further, that hemoglobin and methemoglobin were capable of supporting growth, while hematin and hemin, on the other hand, were effective only in association with other bacteria. This author suggests that hemoglobin acts as a catalytic agent in rendering oxygen available for the bacilli.

In a recent publication on the nature of the effect of blood pigment upon the growth of *B. influenzæ*, Fildes (8) favors the view that the iron-containing pigments function as catalysts in accelerating the transfer of oxygen to the bacilli. In interpreting the relative feebleness of growth on unchanged blood compared

to that on changed blood, Fildes attributes the growth differences to the fact that the oxygen affinity of unchanged hemoglobin may, by its own avidity, divert the oxygen from the bacilli, while physiologically inactive derivatives cannot intercept the oxygen liberated by the catalytic action of the iron in these pigments. In his experiments, however, Fildes never observed the occurrence of growth of *B. influenzæ* in the total absence of blood pigment.

In preceding papers (1–3) the authors have pointed out that the hemophilic bacilli, of which Pfeiffer's bacillus serves as a type, require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (*a*) a vitamine-like substance which can be extracted from red blood corpuscles, and from yeast and vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (*b*) a so called X substance which is also present in red blood cells, is heat-stable and acts in minute amounts.

The present paper concerns itself with the nature of this X substance in blood. It will be shown that this X factor can be derived also from sources other than blood, just as in a previous paper it was shown that the V factor can be supplied apart from animal tissue. That both the V and X factors exist in combination in nature will be shown by the fact that *Bacillus influenzæ* will grow in blood-free medium provided certain plant tissues are added.

EXPERIMENTAL.

Presence of the X Substance in Blood and Blood Derivatives.

In a previous paper (3) it has been pointed out that the X factor, which constitutes one of the growth essentials of *Bacillus influenzæ*, occurs in highest concentration in the cellular fraction of blood, and that the minimal effective amount is extremely small. In fact, the quantity of the X factor carried over in a loopful of the supernatant fluid of a blood broth culture of *Bacillus influenzæ* is sufficient to induce growth in yeast extract broth; that is, in the presence of the supplementing vitamine-like substance, the V factor.

Concentration of Accessory Substances in Blood Essential for Growth of Bacillus influenzæ.—From Table I, in which is shown the minimum concentration of hemoglobin permitting growth of *Bacillus influenzæ* in broth with and without yeast extract, the relationship of the two factors, V and X, is again evident. While the source of the blood

in each instance is different, the two preparations of hemoglobin illustrate in a comparative way the minimal effective ratio between these two factors in the same preparation, and the extraordinarily minute quantity of the X substance which suffices for growth in the presence of an excess of the vitamine-like factor from yeast. The hemoglobin derived from laked blood cells was physiologically active; the crystalline hemoglobin had lost its oxygen-carrying capacity, although it still retained the characteristic absorption bands on spectroscopic examination. The latter preparation was devoid of

TABLE I.

Concentrations of Hemoglobin Essential for Growth of B. influenzæ.

Concentration of hemoglobin in plain broth.*	Hemoglobin† from laked red blood cells.		Crystalline hemoglobin.‡	
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
1 : 10	++	++	—	++
1 : 100	++	++	—	++
1 : 1,000	++	++	—	++
1 : 10,000	+	++	—	++
1 : 100,000	—	++	—	++
1 : 1,000,000	—	+	—	++
1 : 2,000,000			—	+

* All tubes containing 5 cc. total volume were inoculated with 0.05 cc. of yeast extract broth culture of *B. influenzæ*. Plain broth without hemoglobin or yeast extract, and yeast extract broth alone served as controls and showed no growth under the same conditions of seeding.

† Hemoglobin from rabbit blood determined gasometrically by Van Slyke's method (Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127).

‡ Crystals of hemoglobin prepared from ox blood.

++ indicates marked growth; + moderate growth; — no growth.

the more labile vitamine-like substance as shown by the fact that of itself it was incapable of supporting growth even in the highest concentration. When complemented by the V factor in yeast extract, however, a 1:2,000,000 dilution of crystalline hemoglobin sufficed to stimulate growth of *Bacillus influenzæ*. On the other hand, in the medium containing the solution of freshly laked blood cells both factors were present in amounts sufficient for growth up to a certain dilution, beyond which the native V factor was exhausted and the

presence of the residual X substance could be demonstrated only by adding the vitamine principle from yeast. Under the latter circumstances the X factor was still effective in concentrations of hemoglobin as small as 1:1,000,000.

Davis found that hemoglobin from human blood was active in promoting growth of *Bacillus influenzæ* in dilutions as high as 1:180,000. Fildes (8) could not confirm the growth activity of sheep blood in these high dilutions. The sources of error suggested by Fildes, namely the carrying over of blood pigment from tube to tube, or with the inoculum from the culture itself, have been eliminated in the present study by the use of a separate pipette for each dilution, and by seeding all tubes with a small inoculum from a yeast extract broth culture known to contain only the minimal effective dose of the X substance. Furthermore, in the present experiments, broth rather than agar was used as the medium of choice. It seems not impossible, therefore, that these differences in methods, together with recognition of the need of the separation and titration of the two component factors individually, may account for the variations in question. Individual specimens of blood from the same or different species and corresponding derivatives of hemoglobin will of necessity vary in one or the other of these factors, particularly in their content of the V factor, so that comparisons of this sort express only relative values. Of more importance is the quantitative relationship, and the interdependence of these two factors in a given specimen, and the need, in testing for either substance in blood derivatives, of recognizing that the more labile vitamine-like factor may be destroyed in the preparation of the material tested, or lost by dilution without a corresponding destruction or loss of the X substance.

Heat Stability of the X Substance in Blood.—Sufficient data have been presented in the preceding papers of this series to establish the thermostability of the X substance. Unlike the V factor, the X substance in blood, blood extracts, and crystalline hemoglobin resists autoclaving at 120°C. for 45 minutes. Further evidence of the resistance of the X substance to heat is found in the fact that blood charcoal, which reacts positively to the benzidine test, retains the ability to function as the X factor in 10 per cent yeast extract broth; that is, to support growth of Pfeiffer's bacillus in the presence of the supplementing V factor.

Benzidine Reaction.—Olsen (7) states that both the guaiac and benzidine reactions go hand in hand with the ability of blood derivatives to support growth of *Bacillus influenzæ*. This color reaction for the demonstration of the so called peroxidases has been found to parallel the presence of the X substance in blood. The question as to whether the positive benzidine-reacting substances and the X substance in blood are one and the same will be discussed later in describing the occurrence of these substances in plant tissue. It need only be remarked here, that in blood at least, the benzidine-reacting substances exhibit a marked resistance to heat, and in this instance again parallel the behavior of the X substance.

TABLE II.

Absorption by Bone Charcoal of the X Substance in Crystalline Hemoglobin.

10 per cent yeast extract broth 5 cc.* plus	Before absorption.		After absorption.	
	Benzidine test.	Growth-inducing action.	Benzidine test.	Growth-inducing action.
Crystalline hemoglobin, 1 : 1,000.....	++	++	—	—
Bone charcoal.....	—	—	++	++

* Inoculated with 0.05 cc. of 18 hour yeast extract broth culture of *B. influenzæ*.
++ indicates positive benzidine test or marked growth.

Absorption of the X Substance.—It has been noted in the preceding paper of this series that the V factor in yeast extract can be completely removed from solution by absorption with bone charcoal. Similarly it has been found that the X substance can be absorbed from solutions of crystalline hemoglobin by this agent. The solution of hemoglobin after absorption no longer reacts positively to the benzidine test, and has suffered loss of the X substance as evidenced by the lack of any growth-inducing property.

From Table II it is evident that bone charcoal can absorb the X substance from solutions of crystalline hemoglobin. Absorption is facilitated by heat, and is related to the concentration of the X substance in solution and to the time allowed for the reaction. It is of interest to observe that the original bone charcoal failed to give the benzidine test, but, after absorption, reacted positively and had

itself acquired the X substance, as shown by its growth-promoting action in 10 per cent yeast extract broth. The supernatant solution of hemoglobin after absorption had lost both its benzidine-reacting and growth-promoting properties as shown in Table II.

In view of these facts, an attempt was made to determine whether the X substance present in blood might also be found in other proteins of animal origin, such as crystalline egg albumin, or in protein split products such as peptone, erepton, and "aminoids." None of these substances, however, gave a positive benzidine test and none could be substituted for the X factor in supplying the cultural needs of this organism. In these experiments the V factor was always supplied by the addition of yeast extract to the medium, so that if the X factor were present in any test substance, the growth requirements would be complete. Furthermore, lipoidal extracts of heart muscle with and without cholesterol, as described by Noguchi, were tried under similar conditions without result.

Occurrence of the Growth Accessory Substances, X and V, in Plant Tissue.

In the course of these experiments it was observed, as already noted, that a striking parallelism seemed to exist between the presence of the benzidine reaction in blood derivatives and the ability of these same substances to promote growth of *Bacillus influenzæ*. This fact is not final evidence that the reacting substances are necessarily the same in both instances, but suggests rather that this color reaction for the so called peroxidases may serve as an indicator of the presence of the X factor in tissues other than those of animal origin. In the search for the X substance in vegetables, the potato was selected first, since it is known to contain peroxidases and catalysts and also both the fat-soluble A and the water-soluble B vitamines. For these reasons it was thought possible that raw potato would furnish both the V factor and the X substance. This was found to be the case by the luxuriant and continued growth of the bacilli which occurs in blood-free medium containing pieces of sterile raw potato.

Technique.—While no special attempt has been made to devise a precise method for obtaining pieces of sterile potato, and while

modifications will naturally suggest themselves to those trained in bacteriological technique, the following procedure is the one originally adopted. It is desirable to select a potato without surface abrasions or imperfections, and for this reason an old potato is preferable because of the thicker skin protection. Possibly differences in the content of the vitamine and the X substances in new and old potatoes exist, but these have not been determined. After thorough cleansing of the outer surface in running water, the skin is dried and well charred with a red-hot searing iron in a broad band encircling the potato. Through this seared band the potato is cut or broken open and from the inner portions small pieces are removed with a sterile scalpel and placed in sterile petri dishes, care being taken to avoid touching the margins or piercing the outer surface of the potato. The sections removed in this fashion are divided into pieces of suitable size and dropped into tubes of plain broth.

Oxidizing Enzymes of Potato.—It is well known that in plant, as well as in animal tissues, substances concerned with physiological oxidation and reduction are widely distributed. Scrapings from fresh potato exposed to the air rapidly change color as a result of oxidation processes. The benzidine reagent in the presence of hydrogen peroxide gives a blue color when applied to the cut surface of potato, the so called peroxidase reaction. Hydrogen peroxide alone applied in a similar way is rapidly decomposed with the liberation of gaseous oxygen through the action of a catalase in the potato. In studying the oxidase, peroxidase, and catalase of potato and other vegetables, Falk, McGuire, and Blount (9) noted that the enzyme reactions were destroyed by heating to boiling for several minutes. They observed that there was no well defined hydrogen ion concentration for maximum action of the vegetable enzymes, but that on the average the optimal zone lay between pH 7 and 10. According to the work of Cohn, Gross, and Johnson (10) on the isoelectric points of the proteins in certain vegetable juices, the natural hydrogen ion concentration of potato is pH 6 to 7.

Sterile raw potato possesses the property of slowly reducing methylene blue in solutions of phosphates ($\frac{M}{15}$, pH 7.5) from which atmospheric oxygen has been excluded by a vaseline seal. It has been noted

that, in hemoglobin, blood, or yeast extract broth under aerobic conditions, growth appears first in the upper layers of the medium where the oxygen tension is greatest. On the other hand, in broth containing raw potato under seal, growth of *Bacillus influenzæ* occurs early in the depths of the medium about the vegetable. Although the natural reaction of potato is acid (pH 6 to 7), and despite the presence of active enzymes, the use of raw potato in well buffered broth (pH 7.8) does not necessitate further adjustment of the reaction for growth of *Bacillus influenzæ*. In the cultivation of organisms more sensitive to reaction changes, the acidity developing in potato medium requires readjustment.

TABLE III.

Effect of Heat on the Growth Accessory Substances of Potato.

Inoculum, 0.05 cc. of yeast extract broth culture of *B. influenzae*.	Potato in plain broth.				Controls; plain broth.	
	Unheated.		Autoclaved at 120°C. for 45 min.			
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
Type* A.....	++	++	—	++	—	—
" B.....	++	++	—	++	—	—
" C.....	++	++	—	++	—	—
" D.....	++	++	—	++	—	—

* Types A, B, C, and D refer to the biological classification of Stillman and Bourn (Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665).

++ indicates marked growth; — no growth.

The nature and interaction of these vegetable enzymes are too complex and too little understood to warrant any interpretation of their possible significance in the growth of *Bacillus influenzæ* in media containing raw potato. The assumption that they may function as catalysts in facilitating the transfer of oxygen to the bacilli is perhaps justified by the fact that in potato broth, under vaseline seal, growth occurs in the depths of the culture about the vegetable tissue.

Heat Stability of the Accessory Substances in Potato.—From Table III it is evident that potato contains both the X and V factors requisite for growth of *Bacillus influenzæ*, since small pieces of the fresh sterile

vegetable in plain broth suffice to stimulate rapid multiplication. Under these conditions, growth is not conditioned by the carrying over of either factor with the inoculum. Repeated experiments have amply confirmed these observations, and their validity is further established by the fact that growth in unheated potato broth is not limited merely to the first transfer, as is the case in yeast extract broth, but may be continued by loop inoculation from tube to tube.

In studying the effect of heat on the growth accessory substances of potato the interesting fact is brought out that the two factors, X and V, react to temperature in the same manner as the similar substances in blood. The vitamine-like principle is less resistant to heat than the X substance (Table III). Potato broth exposed in the autoclave to a temperature of 120°C. for 45 minutes is no longer capable of supporting growth of *Bacillus influenzæ*. That the V factor is destroyed in the heating and that the more resistant X substance is left unimpaired is shown by the fact that the autoclaved medium can be reactivated by the addition of fresh active yeast extract.

Further evidence that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood for its peculiar nutritive requirements is afforded by the fact that *Bacillus influenzæ* will grow in the complete absence of blood derivatives, meat extractives, and animal peptones. The addition of sterile raw potato to Uschinsky's synthetic medium containing asparagine and ammonium lactate suffices to support growth of *Bacillus influenzæ*. A simpler medium of unheated potato in plain buffer solutions of sodium and potassium phosphate ($\frac{M}{15}$, pH 7.5) fulfills the necessary growth requirements of this organism. It is evident then that potato contains both the V and X substances and that these factors together with the native protein and carbohydrate of potato can replace in media blood pigment and tissue derivatives from animal sources.

In the absence of knowledge of the chemical nature of the X substance and in view of the chemical complexity of the tissues in which it occurs, the identity of the X factor must remain a matter of more or less conjecture. In blood, this substance seems to be associated with the iron-containing pigment, but attempts to substitute inorganic, organic, and colloidal forms of iron have been unsuccessful. The

occurrence of the X factor in potato, as well as in blood, suggests that this substance, or substances reacting similarly, may be commonly present in plant and animal tissue. Other vegetables have not been tested; banana, however, has been used and found to contain the essential growth factors. It is interesting to note that although furnishing the necessary growth accessory substances, banana fails to react positively to the benzidine tests. It possesses, however, a markedly active catalase, as evidenced by the evolution of gas when hydrogen peroxide is applied to the cut surface.

DISCUSSION.

The importance in animal nutrition of the presence in foodstuffs of growth accessory substances is now fully appreciated. There is a growing realization among biologists that this peculiar sensitiveness to the want of some particular substance in small amounts is not limited merely to the higher animals. In microbiology this principle finds its earliest expression in the work of Wildiers (11) (1901) on "bios," a substance extractable from yeast cells which exerts an accelerating influence upon the growth of yeast. Bottomley (12) has extracted from decomposing peat, substances which stimulate plant growth, and to which he has given the name "auximones."

The fact is also gaining recognition that bacteria require for growth not merely carbohydrates, proteins, and their derivatives suitably combined in a medium of optimal reaction. Many of the more difficultly cultivable microorganisms are sensitive to the lack in ordinary media of growth accessory substances allied perhaps to those of animal diet. The application of this principle to culture media has given rise to various modifications in the methods of preparation, each of which seeks to preserve or add certain substances, the presence or absence of which is recognized only by the growth-promoting value of the medium for a particular species. No group of organisms exhibits a more striking sensitiveness in this regard than the so called hemophilic variety.

The observations of Thjötta (2) on the multiplication of Pfeiffer's bacillus in hemoglobin-free medium have demonstrated that substances of bacterial origin can replace the growth-inducing factors of blood. It has been pointed out (1, 3) that there are two distinct and separable

substances in blood essential to the growth of this bacillus—a vitamine-like principle, the so called V factor, and a second substance, the so called X factor. Both of these substances are found in highest concentration in the cellular fraction of blood. They are both requisite for growth of hemophilic bacilli, and each is separately inactive and without effect. They differ in character one from the other in ways suggestive of the nature of their separate functions. The vitamine-like substance, the so called V factor, can be extracted from red blood corpuscles, from yeast and vegetable cells; it is relatively heat-labile, readily absorbed from solution by charcoal, required in greater concentration than the X factor, and resembles the known vitamines in its growth-promoting action. The second substance, the so called X factor in blood, is heat-stable, present in greatest concentration in red blood cells, absorbed from solution by charcoal, and effective in such minute amounts as to suggest a catalytic nature.

This dual function of blood in fulfilling the growth requirements of *Bacillus influenzæ* has been appreciated though less fully analyzed by previous observers. Davis (5), particularly, has directed attention to the fact that for maximum development of hemophilic bacteria both pure hemoglobin and a second vitamine-like substance are essential. He suggests that these two principles in blood may be analogous to the fat-soluble A and water-soluble B vitamines. In the separation of blood pigment from the other constituents of peptic digests of blood, Fildes (8) observed that neither the clear, colorless filtrate nor the precipitated pigment alone could support growth of *Bacillus influenzæ*. Combination of these two factors in media, however, afforded conditions favorable to growth.

Bacillus influenzæ has heretofore been considered an obligate hemophile. The present studies indicate, however, that the hemophilic property of this group of organisms has been based on a lack of knowledge of their essential nutritional needs. Evidence is presented in this paper that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood or blood derivatives for its growth requirements. It has been shown that this organism will grow in the total absence of blood derivatives, meat extractives, and animal peptones. The growth accessory substances which occur in blood have been found to exist also in plant tissue. Sterile raw potato can replace blood in the cultivation of *Bacillus influenzæ*.

On the basis of the facts presented, it seems not unreasonable to assume that nutritional deficiency in the cultivation of other bacteria may be overcome by the addition to culture media of the appropriate growth accessory substances.

CONCLUSIONS.

1. Growth of *Bacillus influenzæ* depends on two distinct and separable substances.
2. These growth accessory substances are present in blood.
3. They occur in plant as well as in animal tissue.
4. Sterile, raw potato will serve as a substitute for blood in the cultivation of *Bacillus influenzæ*.

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* Since the acceptance of this paper for publication the following articles have appeared on the accessory substances concerned in the growth and symbiosis phenomenon of Pfeiffer's bacillus:

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THE DETERMINATION OF THE GASES OF THE BLOOD.

By DONALD D. VAN SLYKE and WILLIAM C. STADIE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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During several years of use certain improvements have developed in the technique for using the apparatus designed by one of us for extracting from the blood and measuring the carbon dioxide (Van Slyke, 1917), oxygen (Van Slyke, 1918), and carbon monoxide (Van Slyke and Salvesen, 1919). A technique has furthermore been developed whereby all the blood gases may be determined in one sample of 1 cc. of blood. The purpose of the present paper is to present these developments.

A Modified Form of Blood Gas Apparatus for Especially Accurate Work.[1]

For analyses in which it is desirable to reduce the error in reading the gas volumes to less than 0.005 cc. an apparatus modified in one respect from the original macro form (Van Slyke, 1917) is now used. The upper, graduated stem of the apparatus has been reduced in bore

[1] The apparatus, with the water jacket and mechanical shaking device, may be obtained from Emil Greiner, 55 Fulton Street, New York.

from 4 mm. to 2.7 mm., so that its cross-section is halved. Consequently the length of tube holding 1 cc. of gas is lengthened from 75 to 80 mm. to 150 to 160 mm. and it is divided into 100 instead of 50 divisions. In the finer tube gas volumes can be estimated to 0.1 of a division, or 0.001 cc. Also, because of its narrowness the column of liquid can be easily seen through and the meniscus read sharply, even when the solution is deeply colored, as in whole blood analyses.

The only difference in manipulation necessitated by the change in construction is in the manner in which the apparatus is shaken in extracting the gases. The extraction is accomplished by whirling the blood mixture about the wall of the chamber, rather than by the repeated inversion of the apparatus. The measuring tube is of such small diameter that liquid caused to lodge in it by inverting the apparatus is dislodged with some difficulty. The mechanical shaker described by Stadie (1921) is well adapted for use with this form of the apparatus.

In the narrow tube CO_2 and O_2 may be absorbed by admission of alkali and pyrogallol solutions as readily as in the wider tube of the original apparatus. It is desirable, however, in admitting the absorbing solution from the cup at the top of the apparatus to open the cock so slightly that the solution trickles down only one side of the narrow measuring tube below. If the solution enters so rapidly that it forms a solid column in the measuring tube it is dislodged by tapping the tube with the finger, or by running in mercury from the cup. When a viscous absorbing solution, like pyrogallol, is used, it is followed by a little water to clear the inner wall of the measuring tube. Even after careful admission of water or absorbing solution it is the rule for a slight but measurable amount to stick in the upper end of the measuring capillary, just below the stopcock. A few gentle taps with the finger, or the admission of a drop of mercury from above, suffice for dislodgement.

After the admission of any fluid from the cup into the chamber, time should be allowed for drainage before the gas volume is read. A few seconds suffice if only 0.1 cc. of gas remains to be measured, but 2 minutes are required if the volume approaches 1 cc.

In order to obtain the benefit of the accuracy obtainable with the fine bore apparatus, it is necessary to use it in a room in which

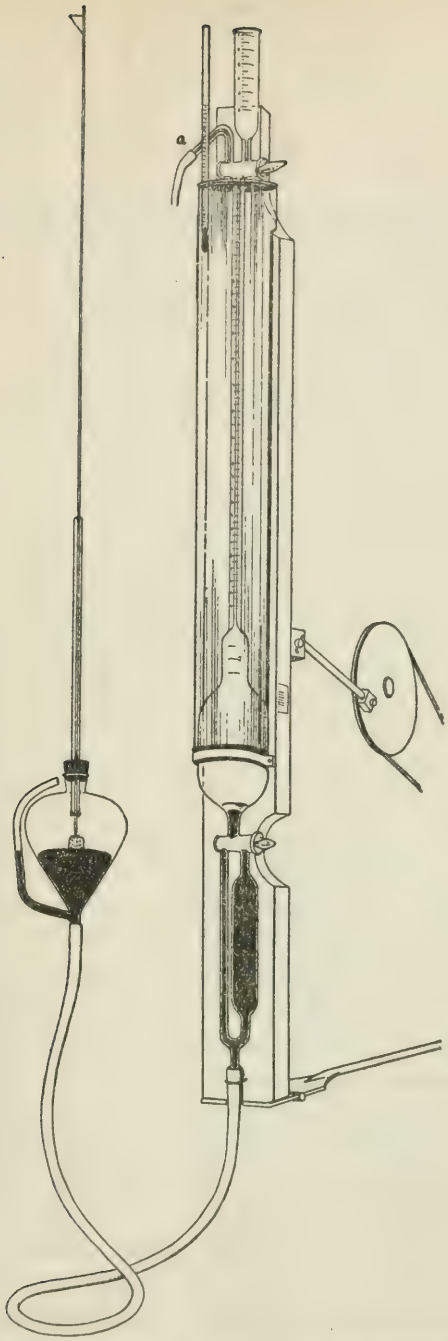

FIG. 1. Fine bore apparatus with water jacket and shaking device. A Stadie scale for use in oxygen determination is shown marked on the side of the supporting board. The stick projecting from the levelling bulb is for measuring 500 mm. reduction in pressure. It is well to insert a glass air trap between the apparatus and levelling bulb, as described for the micro-apparatus (Van Slyke, 1917, p. 363).

the temperature does not vary by more than 1°C., or to provide it with a water jacket such as is shown in Fig. 1. The apparatus may be shaken by hand, or may be attached to a hinged board, as shown in Fig. 1, so arranged that the shaking may be done by a motor.

The manipulation of the apparatus with the finer bore requires slightly more time and care than that of the original apparatus, and the latter is adequate for most purposes, such as the determination of the oxygen capacity of blood as a measure of the hemoglobin, and of the carbon dioxide as a measure of the alkaline reserve, both values being capable of determination by the original apparatus to within 1 per cent of the amounts normally measured. For some purposes, however, such as the determination of all the gases in 1 cc. of blood, the determination of carbon monoxide, or of the slight amount of nitrogen gas in blood, or when it is essential to keep the error in CO_2 determinations below 0.5 volume per cent, or in oxygen determinations below 0.25, the fine bore apparatus is desirable.

In calibrating the apparatus, we attach to the bottom, either by sealing or by joining glass on glass with a heavy-walled piece of suction tubing, a glass tube bearing a fine bore stop-cock and drawn out into a fine capillary. Through the latter water is drawn up into the apparatus by means of suction applied to the outlet above the upper cock. When the entire apparatus and the upper outlet are filled with water, the cock of the capillary attached below is closed, and the suction is discontinued. The upper cock of the apparatus is then turned, so that the chamber is connected with the empty cup. The water is now delivered through the cock of the attached capillary, 0.1 cc. at a time. The drops are caught in a weighing bottle containing a layer several mm. thick of paraffin oil. After the delivery of each drop, the tip of the capillary is touched to the surface of the oil, to detach all adhering water except a uniform minimum. The drops of water sink beneath the oil, and loss of weight by evaporation is prevented with completeness that is not obtainable by merely using a covered weighing bottle, which must be opened to receive each addition of water. The weighings are made to the nearest milligram.

The calibration may also be performed with mercury, as described

for the micro-apparatus.[2] In this case sealing the delivery capillary to the apparatus is necessary, as the pressure is sufficient to expand appreciably a rubber tube.

The constancy of results which may be obtained with the fine bore apparatus is indicated by Table I, which shows the duplicate readings obtained in a series of analyses performed by Dr. J. P. Peters, Jr., in determining the CO_2 absorption curve of a specimen of blood.

TABLE I.

Results of Series of Duplicate Determinations of CO_2 in Whole Blood with the Fine Bore Apparatus.

1 cc. of blood was used for each analysis.

No.	*V.* Total volume of gas extracted.	$V - CO_2$. Observed volume after absorbing CO_2 with NaOH.	CO_2 By difference.
	cc.	*cc.*	*cc.*
1	0.735	0.042	0.693
	0.725	0.033	0.692
2	0.604	0.130	0.474
	0.603	0.130	0.472
3	0.704	0.128	0.576
	0.696	0.122	0.574
4	0.763	0.126	0.637
	0.764	0.126	0.638

Magnification of Small Gas Volumes by Reduction of Pressure.

When an accurate measurement of very small gas volumes (*e.g.*, less than 0.05 cc.) is desired, as may be the case in determining the N_2 or CO content of blood, it is desirable for measurement to increase the gas volume by reducing the pressure. The reduction is accomplished by holding the levelling bulb lower, by the desired distance, than it would be placed in order to put the gas in the apparatus under atmospheric pressure. We have found 500 mm. of mercury to be a

[2] Van Slyke (1917), p. 365.

convenient reduction in pressure for this purpose. For readily locating this level we have used the light rod with the marker attached near its top, shown projecting up out of the levelling bulb in Fig. 1. The rod is a pine stick 2.5 mm. in diameter and a little over 500 mm. long. The marker is a piece of stiff paper glued on 501 mm. from the bottom. The stick passes down into the levelling bulb through a piece of glass tubing about 10 cm. long, which is held upright in a rubber stopper and serves to keep the stick in a vertical position during readings. At its lower end, within the levelling bulb, the stick terminates in a cork, which floats on the mercury. It dips 1 mm. below the surface in the case of our apparatus (the exact distance would, of course, vary for different sticks and corks) and to correct for this depth of immersion the distance from the bottom of the cork to the marker is made 501 instead of 500 mm.

The measurement is made merely by placing the bottom of the marker at the level, in relation to the mercury and water levels inside the apparatus, at which one would place the level of the mercury itself in the bulb in order to obtain atmospheric pressure within the apparatus.

The volume of gas read is reduced to atmospheric pressure by multiplying by the factor $\frac{B-H-w}{B-w}$, B being the atmospheric pressure H the height from the mercury in the levelling bulb to the marker on the stick (*e.g.*, 500 mm.), and w the vapor tension of the water at the temperature at which the reading is taken.

For example, a gas volume is read as 0.133 cc. at 25°, and 765 − 500 = 265 mm. pressure. The vapor tension of water at 25° is 23.6 mm. Hence, the volume of gas measured moist at room temperature and pressure would be $0.133 \times \frac{265 - 23.6}{765 - 23.6} = 0.0433$. This is reduced to 0°, 760 mm. measured dry by the usual factor given in Table XIII, which in this case is $0.888 \times \frac{765}{760} = 0.894$. The consequent volume at 0°, 760 mm. is therefore $0.0433 \times 0.894 = 0.0387$ cc.

The Nitrogen Gas Content of Blood, and its Effect on the Calculation of Results of Other Blood Gases.

In the original paper on the determination of oxygen, the oxygen and nitrogen were extracted from the blood and measured together, and the oxygen was estimated by deducting the relatively small amount of nitrogen, which was calculated from Bohr's determinations (1905) of the solubilities of gases in blood and water. The solubility coefficient of nitrogen gas in water (the volume of gas reduced to 0°, 760 mm., which 1 volume of water dissolves when in equilibrium with the gas at 760 mm. tension) at 38° is 0.0122; in blood Bohr found the solubility coefficients of chemically inert gases to be 92 per cent as great as in water. The volume of nitrogen calculated to be held in solution by 100 cc. of water at body temperature and 760 mm. barometric pressure (diminished by 48 mm. vapor tension of water) in equilibrium with the atmosphere is, therefore, $100 \times 0.0122 \times 0.791 \times \frac{760-48}{760} = 0.90$ cc. For blood, the calculated amount would be 0.92 as great, or 0.83 cc.

Earlier analysts had found as much as 4 to 5 volumes per cent of nitrogen gas in the blood, and attributed the excess above that which water would dissolve as due to leakage of air into the evacuated apparatus used.

Bohr and Henriques (1897) by more accurate methods determined the nitrogen gas, along with the carbon dioxide and oxygen, in both arterial and venous bloods of a number of dogs (twenty-two analyses). They found that the nitrogen contents varied from the theoretically expected 0.8 volume per cent to 1.7 volumes per cent, the average and most of the individual figures being in the neighborhood of 1.2 volumes per cent. In a paper published at nearly the same time Bohr (1897) found that when shaken with air at room temperature blood of the ox and dog, and likewise 10 to 12 per cent solutions of oxyhemoglobin, absorbed 1.65 to 1.98 volumes per cent of nitrogen. Water under the same conditions absorbed only 1.30 volumes per cent. Peculiarly enough, oxygen-free nitrogen was absorbed in no greater proportion by blood than by water. For the fact that blood in contact with air absorbs more nitrogen than does water, there appears to be as yet no well grounded explanation, although Bohr suggested that some easily dissociated oxide of nitrogen might be formed when nitrogen and oxygen meet in the presence of dissolved hemoglobin. In Bohr's later paper (1905) on the absorption coefficients of gases in blood and water the bearing of these results on the absorption coefficient of nitrogen in blood is not considered, and the coefficient is given as 92 per cent of that in water.

When the original paper on the determination of oxygen in the blood was published (Van Slyke, 1918) Bohr's earlier data (1897) were overlooked, and the corrections for nitrogen gas were based on the solubility coefficient given in his 1905 publication. Van Slyke and Salvesen (1919), in later analyses in which the oxygen and nitrogen were measured separately, the oxygen being absorbed in the apparatus by pyrogallol, found that the nitrogen gas in rabbit blood approximated 1.2 volumes per cent, a figure which agrees with the average found in dog blood by Bohr and Henriques (1897). Smith, Dawson, and Cohen (1919–20) have recently reported further analyses showing a nitrogen content of blood higher than that estimated from the solubility coefficient.

We have performed a number of determinations of the nitrogen gas content of blood, both as drawn from the veins and after equilibration with air at room temperature. It was found that the same results were obtained when no reagents were added as when ferricyanide was used to release the oxygen, or acid to release carbon dioxide. Consequently in the series here reported the nitrogen was determined by extraction in a vacuum without the addition of reagents. The apparatus employed was the model with the finer bore measuring tube described at the beginning of this paper. The technique used was the following:

3 cc. of water and a few drops of octyl alcohol were freed completely from air by vacuum extraction in the apparatus in the usual manner. The extraction was always repeated in order to make certain that not even 0.001 cc. of air was left. 1 to 2 cc. of the water were run up into the cup, and 5 cc. of fresh blood, which had been drawn under paraffin oil without exposure to air, were run under the water and into the chamber of the apparatus. (The fact that the water during its brief contact with air in the cup absorbed no measurable amounts of air was proved by controls.) The blood-water mixture was evacuated in the apparatus and the gases were shaken out by hand for 3 minutes; longer shaking was found not to increase the yield of nitrogen gas. After release of the vacuum the oxygen and carbon dioxide were absorbed by allowing a few drops of alkaline pyrogallol solution to run slowly from the cup down the measuring tube. When absorption was complete, as shown by shrinkage of the gas to a constant volume, the somewhat viscous pyrogallol solution was washed from the inner walls of the tube by means of a little water, and the volume of unabsorbed gas was measured as nitrogen. The results are given in Table II.

The results in Table II confirm those of Bohr in showing that the blood, both in the veins and after aerating at room temperature, contains about 0.5 volume per cent more nitrogen gas than calculated

from the solubility coefficient of the gas in water. Our results, perhaps because the simplicity of our method has reduced the chance of error, vary over a smaller range than Bohr's. In venous blood the total range is 1.36 ± 0.11 volumes per cent of nitrogen, while in blood aerated at 23–30° *in vitro* it is 1.52 ± 0.2 volumes per cent.

TABLE II.

Nitrogen Gas in Whole Blood.

No.	Subject.	Duplicates.		Mean.	Temperature.
Venous blood as drawn.					
		vol. per cent	*vol. per cent*	*vol. per cent*	°C.
1		1.36	1.42	1.39	
2	St.	1.30	1.39	1.35	
3	M. L.	1.63	1.30	1.47	
4	Kel.	1.33		1.33	
5	Schu.	1.36		1.36	
6	M. S.	1.30		1.30	
7	Koh.	1.43		1.43	
8	Dog 1.	1.44	1.26	1.35	
9	" 2.	1.31	1.40	1.36	
10	Dr. B.	1.25		1.25	
Average				1.36	
Venous blood saturated with air at room temperature.					
1	E.	1.78	1.63	1.71	24
2	St.	1.47	1.64	1.56	23
3	Schu.	1.30	1.31	1.31	29
4	Koh.	1.52		1.52	25
5	V. S.	1.60	1.60	1.60	23
6	Dog 2.	1.40	1.41	1.41	25
7	Ox.	1.55		1.55	30
Average				1.52	

The results in Table II, as well as those of Bohr above referred to, include as "nitrogen" all the gas extracted by evacuating blood and left after absorption of oxygen and carbon dioxide. Regnard and Schlœsing (1897) found besides nitrogen 0.04 volume per cent of argon in this gas fraction from horse blood, 2.00 volumes per cent still remaining and being calculated as nitrogen. Whether it actually

is all nitrogen, or a mixture of nitrogen and gases, such as methane, hydrogen, etc., not yet identified, is still uncertain. Hartridge (1919–20) has found as much as 1 volume per cent of carbon monoxide in the blood of a heavy tobacco smoker, but there appears to be no reason to believe that carbon monoxide forms any part of the unexplained "nitrogen" ordinarily present in normal blood. The probability that the residual gas is actually nitrogen is strengthened by the fact that it is increased by aerating the blood at room temperature. The reverse would be expected if any considerable proportion of the residual gas were other than nitrogen.

It is evident from the above that when the nitrogen and oxygen, or nitrogen and carbon monoxide, of the blood are measured together, the volume of the gas accompanying the nitrogen cannot be estimated by deducting the volume of nitrogen estimated (as 0.83 volume per cent of the blood) from its solubility coefficient, without introducing an error of approximately plus 0.5 volume per cent into the calculation of the oxygen or carbon monoxide.

There are two alternatives. (1) The average N_2 content of blood as empirically determined (Table II) may be subtracted from the $N_2 + O_2$ or from the $N_2 + CO$ in order to estimate the O_2 or the CO. (2) The gas measured with the nitrogen may be determined by absorption with a proper reagent (*e.g.*, pyrogallol for oxygen). The details of the calculations, from analyses made by both alternatives, of oxygen content, oxygen capacity, carbon monoxide content, and of methemoglobin, the determination of which is based on oxygen capacity determinations, will be taken up in the following pages in connection with the discussions of these determinations.

Determination of Oxygen.

1. Changes in Technique.

A. Reduction in Amount of Ferricyanide Used.—We have reduced the amount of potassium ferricyanide per cc. of blood from 60 or 70 mg. (0.4 cc. of saturated solution for 2 cc. of blood) to 10 mg., which, as shown in Table III, is still twice the minimum necessary amount. The ferricyanide solution used contains 20 gm. per 100 cc. Of this 0.1 cc., containing 20 mg. of the salt, is used for 2 cc. of blood.

The smaller amount of ferricyanide causes as rapid and complete an evolution of oxygen as do larger amounts, and it has two advantages over the latter. First, the smaller amount does not appreciably retard laking, and it is consequently unnecessary to wait for laking to become complete in the apparatus before the ferricyanide is introduced. Also, the possibility of error from incomplete laking is avoided. Second, reduction in the amount of ferricyanide reduces the formerly somewhat annoying amount of precipitate formed by interaction with the mercury in the apparatus.

B. Use of Water Instead of Ammonia for Laking Blood.—In the original procedure (Van Slyke, 1918) the blood, before addition of

TABLE III.

Effect of Varying Amounts of Potassium Ferricyanide on the Volume of Oxygen Liberated from 2 Cc. of Normal Human Blood.

Ferricyanide added to 2 cc. of blood.	$O_2 + N_2$ measured at 20°, 765 mm.	Calculated O_2 content of blood.*
gm.	*cc.*	*vol. per cent*
0.005	0.41	17.3
0.010	0.53	22.8
0.020	0.53	22.8
0.100	0.53	22.8

* 1.36 volumes per cent of N_2 being subtracted from $O_2 + N_2$ after reduction of the total volume to 0°, 760 mm.

ferricyanide, was laked in the apparatus with saponin in a dilute ammonia solution, the proportions of blood and ammonia being those previously adopted by Haldane and by Barcroft in their well known methods for determination of blood oxygen.

We have found that the use of such a mixture may introduce two errors, both usually small but frequently measurable by our present technique: (1) the alkalinity of the mixture is not sufficient entirely to prevent in all cases the escape of CO_2, particularly when the room temperature is high, and (2) the alkaline reaction accelerates some oxidative process by which part of the oxygen freed is slowly consumed. Hence, as shown by Table V, when the oxygen is freed in too alkaline a solution, the entire amount is not obtained.

With the dilute ammonia formerly used, the slight alkalinity caused but little oxygen consumption, sometimes none (Table VI) and the average error from this source was approximately balanced by the error in the opposite direction due to the small amount of CO_2 set free. Consequently the normal figure for the oxygen capacity of human blood determined by the former technique is not affected by present changes.

At a temperature of 20°, and with ammonia of at least 0.06 N concentration (obtained by diluting 1 cc. of ordinary concentrated ammonia to 200 cc.), the CO_2 tension is usually too near zero to affect measurably the volume of the extracted gases (no decrease observed when the gases are washed with NaOH solution). If the temperature rises to 25 or 30°, however, or if by reason of standing or preparation from a weak stock solution the ammonia is of less than usual concentration, enough CO_2 may escape with the O_2 to increase the calculated volume per cent of the latter by several tenths, and occasionally more. There is also a difference in bloods, some freeing measurable amounts of CO_2 under conditions where others do not. Presumably bloods richer in hemoglobin, which acts as a weak acid, require more alkali to reduce their CO_2 tension to zero.

The obvious way to prevent mixture of CO_2 with the $O_2 + N_2$ would be to increase the alkali added to the blood to such an extent that there would in all cases be a sufficient excess. Such addition, however, increases the error due to oxygen consumption.

The latter error, like that due to CO_2 escape, may be nil or slight within the 2 minutes that usually suffice to extract all the oxygen. It is not uncommon, however, on extracting for a third minute, to observe a decrease of a few thousandths of a cc., amounting to 0.1 to 0.3 volume per cent of the blood.

At present, therefore, we use water for laking the blood, which seems to obviate the error due to oxygen consumption. The 10 to 20 volumes per cent of CO_2 that accompany the oxygen are removed after the extraction by absorption with NaOH solution, before the $O_2 + N_2$ volume is measured. Consequently the danger of error from admixture of CO_2 with the $O_2 + N_2$ is also avoided.

The avoidance of ammonia has an additional advantage in reducing the insoluble black precipitate which forms in its presence by the

interaction of ferricyanide and mercury. With water as diluent and the lesser amounts of ferricyanide now used the precipitate is reduced to practically nil.

C. Details of Present Oxygen Determination.—The apparatus is washed out twice before each analysis with water, in the rapid manner described later in connection with the CO_2 determination (p. 548), in order to remove the alkali used to absorb CO_2 in any previous analysis. For 2 cc. of blood 6 cc. of water, 0.3 cc. of 1 per cent saponin (Merck) solution, and 2 or 3 drops of caprylic alcohol are introduced into the apparatus and freed of air, as described in the original paper (Van Slyke, 1918), the extraction being repeated until no more air is obtained.

Nearly the entire 6 cc. is then forced up into the cup of the apparatus. The blood is stirred to assure even distribution of corpuscles, and drawn into a pipette calibrated to *deliver* 2 cc. between two marks, of which the lower is 3 or 5 cm. from the tip. The pipette is introduced under the water solution in the cup, so that the tip rests on the bottom near the capillary. As the blood flows out of the pipette held in the left hand, the stop-cock is partially opened with the right, so that the blood accompanied by some of the water, flows at once on into the chamber of the apparatus. The layer of blood need never rise more than 1 or 2 mm. above the bottom of the cup, and the slight amount adhering is washed completely into the chamber by the water which follows after all the blood has been delivered.

Before the last cc. of water is readmitted, 0.10 to 0.12 cc. of a solution, containing 20 gm. of potassium ferricyanide per 100 cc., is added and thereby introduced into the chamber after the blood. (The ferricyanide may be measured with sufficient accuracy as 3 drops from a dropper which delivers 1 cc. in 25 to 30 drops.) A mercury seal is made, and the apparatus is evacuated and shaken for 3 minutes. Usually 2 minutes, frequently less, are sufficient, but repeated trials have shown that 3 minutes are sometimes required before the last traces of oxygen are extracted. Owing to the continued slow evolution of CO_2 from the unalkalinized mixture, it is not possible to check the completeness of the $O_2 + N_2$ extraction by repeated measurements of the $O_2 + N_2 + CO_2$ volume; so it is desirable to continue the extraction in each case for a sufficient period to insure

its completion. Extraction being complete, the vacuum is released by opening the lower cock, and the mercury together with the reaction mixture is allowed to run up into the chamber of the apparatus. The gases obtained from 2 cc. of blood are normally about 0.50 cc. of O_2, 0.015 cc. of N_2, and 0.2 cc. of CO_2, about one-fifth of the total blood CO_2 being evolved under the conditions of the analysis. In order to absorb the CO_2 the levelling bulb is placed at such a height that the mercury in it is slightly below the level of the mercury in the apparatus, so that a slight negative pressure is maintained in the latter. 0.5 cc. of 0.5 N NaOH solution, previously saturated with air or oxygen, is admitted from the cup of the apparatus and allowed to trickle slowly down the inner wall to absorb the CO_2 from the gas mixture. If, as is frequently the case with the fine bore apparatus, the latter part of the solution enters as a solid column instead of running down the walls, it is dislodged by letting a little mercury pass down through it in a fine stream from the cup above. Since the mercury droplets are coated with alkali solution, they rapidly complete the absorption of CO_2 as they pass the gas column.

Even when all the alkali solution appears to flow down the sides of the gas pipette a little usually remains just below the stop-cock. If neglected, this amount introduces an error of from 0.005 to 0.010 cc. in the gas volume reading, but it can be easily dislodged by a drop of mercury run in as above described.

After absorption of CO_2 is complete time for drainage of the alkali solution must be allowed before the reading is taken (see p. 528).

For the reading the height of the levelling bulb is regulated by the scale described by Stadie (1921) in the accompanying paper, and shown in Fig. 1 of this paper. The use of the scale may be obviated by trapping the extracted solution in the bulb below the lower cock, as in the determination of CO_2 (Van Slyke, 1917), before the vacuum is released.

2. Calculations Connected with Oxygen Determinations.

A. Calculation of Total Oxygen Content of Blood.—The total oxygen content of blood includes both the oxygen chemically bound by hemoglobin and that held in physical solution by the blood fluids.

When the oxygen and nitrogen are extracted and measured together,

as described in the original paper, the calculation is made as there described, except that instead of subtracting the theoretically estimated 0.9 volume per cent of nitrogen from the $O_2 + N_2$ content the empirically determined *1.36 volumes per cent are subtracted.*

When from the mixture of $O_2 + N_2$ the O_2 is directly determined by absorption with pyrogallol, as described by Van Slyke and Salvesen (1919), the empirical correction for nitrogen is, of course, avoided. Since absorption is completed in 1 minute it adds but little to the time required for an analysis. It is a useful check whenever there is reason to suspect leakage of air into the apparatus, as such an occurrence greatly increases the residual nitrogen gas.

B. Calculation of Oxygen Combined with Hemoglobin in Blood as Drawn.—The gases obtained together as described by extraction of arterial or venous blood collected out of contact with air are: (1) oxygen chemically combined with hemoglobin, (2) oxygen physically dissolved in the blood, and (3) nitrogen physically dissolved. For dissolved nitrogen the value as empirically determined above is 1.36 cc. per 100 cc. of blood. For oxygen the volume physically dissolved depends upon the partial pressure of oxygen with which the blood is in equilibrium. Arterial blood normally is in approximate though not complete equilibrium (Krogh and Krogh, 1910) with the alveolar air, which varies in its oxygen content from 14.5 to 17.5 per cent. Assuming a mean value of 16 per cent, the oxygen dissolved at 38°C. is approximately $100 \times 0.022 \times 0.16 \times \frac{760 - 49}{760} = 0.33$ volume per cent. In venous blood, however, and in partially saturated arterial blood, the amount of physically dissolved oxygen varies with the varying oxygen tension, which is approximately indicated by the percentage of complete saturation of the blood with oxygen. An average value for the percentage of saturation of venous blood is 65 per cent, which corresponds to an oxygen tension of 35 mm. when the CO_2 tension is normal (40 mm.). There is, therefore, $100 \times 0.022 \times \frac{35}{760} = 0.10$ volume per cent of oxygen physically dissolved in such a sample.

Therefore when the total O_2 is determined by extraction and absorption with pyrogallol, the O_2 combined with hemoglobin may

be approximately estimated by subtracting from the total O_2 0.3 volume per cent for arterial blood and 0.1 for venous.

When N_2 and O_2 are measured together, the corrections for dissolved $N_2 + O_2$ which are subtracted in order to estimate the combined O_2 are $1.36 + 0.3 = 1.66$ volumes per cent for arterial, and $1.36 + 0.10 = 1.46$ for venous blood. Discarding the last decimals, the corrections are 1.7 volumes per cent for arterial blood, 1.5 for venous.

The above corrections are estimated from the average oxygen tensions of arterial and venous blood. The corrections due to dissolved oxygen are so minute that fluctuations from the average, can exceed the experimental error only in most refined work. If, however, a greater degree of accuracy is desired the values in Table IV may be used. They are calculated from the curve of Barcroft (1914).

C. Oxygen Combined with Hemoglobin in Blood Artificially Saturated with Air (Oxygen Capacity of Blood).—In determining the oxygen capacity (usually as a measure of the hemoglobin content) blood is saturated with air at room temperature, as described in the original paper (Van Slyke, 1918). From the total $N_2 + O_2$ obtained on extraction of blood thus saturated, it is necessary to deduct the N_2 and the physically dissolved O_2. According to Bohr (1905) the solubility coefficient of oxygen in blood is 0.031 at 15°, 0.022 at 38°. For the usual room temperature range of 15–25°, the coefficient varies from 0.031 to 0.027, with a mean of 0.029 at 20°. The volume per cent of oxygen physically dissolved by blood equilibrated with atmospheric air at 20°, 760 mm., may therefore be estimated as $100 \times 0.029 \times 0.209 \times \frac{760 - 17}{760} = 0.59$. At 25° the figure would be 0.55, at 15° it would be 0.63. The figure thus calculated from the solubility coefficient may, as in the case of nitrogen, not be exact, but it does not appear probable that the error, if there is one, is large enough to be significant. If we add the figure for oxygen dissolved at 20° to the empirically determined nitrogen content of blood saturated with air at room temperature we have $0.59 + 1.52 = 2.11$ volumes per cent of physically dissolved $O_2 + N_2$ to subtract from the total $O_2 + N_2$ content in order to obtain the oxygen combined with hemoglobin.

When the total O_2 of the saturated blood is determined directly,

by absorption with pyrogallol, correction is to be made only for the 0.59 volume per cent of physically dissolved oxygen.

In Table I of the original article (Van Slyke, 1918) the volume of "dissolved air" to be subtracted from blood shaken at 20° was calculated from the solubility coefficient of air to be 0.034 cc. This corresponds to 0.031 cc. measured at 0°, or 1.55 volumes per cent of the 2 cc. of blood analyzed, a figure 0.55 volume per cent below that

TABLE IV.

Oxygen Estimated Held in Physical Solution by Blood at 38° at Different Oxygen Tensions and Saturations.

Saturation of blood with oxygen.	Oxygen tension corresponding to saturation in Column 1.	Oxygen physically dissolved at 38°C.	Total correction $N_2 + O_2$ (dissolved).
per cent	*mm. Hg*	*vol. per cent*	*vol. per cent*
0	0	0.00	1.36
10	11	0.03	1.39
20	16	0.04	1.40
30	19	0.05	1.41
40	22	0.07	1.43
50	27	0.08	1.44
60	31	0.09	1.45
70	37	0.11	1.47
80	46	0.13	1.49
90	65	0.18	1.54
95	90*	0.24	1.60
100	120	0.32	1.68
100	140†	0.37	1.73
100	150‡	0.40	1.76

* Approximate arterial tension.

† Tension of blood equilibrated *in vitro* at 38° with air containing CO_2 at 40 mm.

‡ Tension of blood equilibrated *in vitro* at 38° with air containing no added gas.

based on the nitrogen content which we find by actual analysis. Consequently oxygen capacities calculated as outlined in the original paper have been approximately 0.55 volume per cent too high. This corresponds to an error of plus 0.41 gm. of hemoglobin per 100 cc. of blood, or plus 3.0 per cent of the average normal hemoglobin content.

D. Calculation of the Oxygen Unsaturation of Blood.—The oxygen unsaturation of blood was first defined by Lundsgaard (1918) as

the difference between the oxygen content of blood and the oxygen capacity. In determining the oxygen unsaturation Lundsgaard and others, including the authors, have estimated the oxygen capacity as the oxygen content of blood saturated with air at room temperature. At average atmospheric conditions (20° and 143 mm. oxygen tension) blood containing sufficient hemoglobin to bind 20.00 volumes per cent of oxygen would contain 20.58 volumes per cent of total oxygen, the extra 0.58 being the amount held in physical solution. However, at 38° the same blood would likewise bind 20.00 volumes per cent of oxygen with its hemoglobin, but would dissolve physically only 0.32 volumes per cent, making a total of 20.32 volumes per cent. The oxygen unsaturation of such a blood would be calculated according to the above mentioned method as 20.58 − 20.32 = 0.26 volume per cent, instead of zero, which it obviously is. The error of calculation is barely within the limit of experimental error of the determinations, and is altogether too slight to affect significantly any results thus far published, but it seems nevertheless worth while to correct it in the future by basing the calculations on figures representing oxygen combined with hemoglobin rather than on total oxygen. The example given at the end of the paper indicates the preferred mode of calculation.

Table XIII at the end of this paper will be found of convenience in making the above calculations.

3. Magnitudes of Error in Different Steps of the Oxygen Determination.

A. Measurement of Blood Sample.—The accuracy with which samples of blood can be delivered from an Ostwald pipette of the type described by Van Slyke and Cullen (1914, p. 215) is indicated by the following successive weighings. The blood was well stirred before each sample was drawn. Nine successive weighings from a 2 cc. pipette gave 2.1072, 2.1090, 2.1100, 2.1076, 2.1093, 2.1071, 2.1072, 2.1051, and 2.1069 gm. of blood, the maximum variation being from 2.1101 to 2.105, or 2.1075 ± 0.0025 gm., the maximum deviation, 0.0025 gm. being 0.11 per cent, the average 0.05 per cent of the amount measured.

B. Reading of Gas Volume in Fine Bore Apparatus with 0.1 to 0.2

Cc. of Water above Mercury. Levelling Bulb Adjusted with Eye. No Side Arm or Levelling Scale.—A series of measurements on the same portion of gas under the above conditions gave, when reduced to 760 mm., 0°, eight successive readings between 0.729 and 0.732 cc., the maximum deviation being, therefore, 0.0015 cc. from the mean, or 0.2 per cent of the gas volume measured.

C. Reading of Gas Volume in Fine Bore Apparatus with 8.5 Cc. of Water above Mercury. Side Arm on Levelling Bulb. New Levelling Scale (Stadie, 1921) Used in Adjusting Levelling Bulb.—Nine successive readings on a given gas sample varied between 0.884 and 0.882 cc., the maximum deviation being, therefore, 0.001 cc. from the mean, or 0.1 per cent of the gas volume measured. Without the Stadie levelling scale, a maximum variation of ± 0.004 cc., or four times as great was encountered.

D. Reabsorption of Air by Reaction Mixture During Delivery of Blood.—When, after being freed of air, the reaction mixture is forced up into the cup it is exposed to the atmosphere for a period of 1 to 4 minutes while the blood is being stirred and pipetted into the apparatus. Repeated tests have shown that no measurable amounts of air are absorbed by the reaction mixture during even longer periods of quiet exposure to the atmosphere.

E. Constancy of Results Obtainable.—With the use of the levelling scale and the technique described above the volumes of $O_2 + N_2$, reduced to 0°, 760 mm., obtained from 2 cc. samples of blood were the following:

Blood 1, 0.555, 0.555, 0.555, and 0.556 cc.

Blood 2, 0.436, 0.436, 0.442, 0.433, and 0.436 cc.

The first series represents somewhat exceptional constancy, but the second, with a maximum variation of 0.437 ± 0.005 cc., or ± 0.25 volume per cent of oxygen, indicates rather greater variations than are usually to be expected.

4. Relative Yields of Oxygen from Blood Laked in Alkali, Water, and Acid.

The fact that a sufficient grade of alkalinity diminishes the yield of oxygen and that acid may to a less extent cause a similar diminution is indicated by the data in Table V. The analyses were performed

as described on page 539 of this paper, except that instead of 6 cc. of water added to the 2 cc. sample of blood, an equal volume of a solution containing the indicated amounts of acid or alkali was added. The extracted gas was tested with NaOH solution to absorb CO_2 after use of the alkaline as well as the acid and neutral solutions. An amount of CO_2 equivalent to 0.5 volume per cent of the blood was obtained from the blood + ammonia mixture, and to 0.65 from the carbonate.

TABLE V.

Oxygen Content of Blood Determined after Laking in Alkali, Water, and Acid, Respectively.

Alkali or acid added.	Amount of acid or alkali per cc. of blood.	$O_2 + N_2$.		CO_2.
	millimols	*vol. per cent*	*per cent of maximum*	*vol. per cent*
Na_2CO_3	0.28	25.15	93.6	0.55
NH_3	0.15	26.35	98.1	0.65
NaOH	0.04	21.30	79.3	
NaOH	0.02	26.40	98.3	
NaOH	0.01	26.85	100.0	
None	0.00	26.80	99.8	
Lactic acid	0.01	26.85	100.0	
" "	0.06	26.65	99.3	
" "	0.10	26.35	98.1	

The results with this blood seem to indicate that the maximum yield of oxygen is obtained when water with negligible amounts of acid or alkali is used. That other bloods do not consistently yield higher oxygen contents when *no* acid or alkali is added is indicated by Table VI, however.

That the variations in results with the different reagents were not due to analytical errors appears to have been excluded by the constancy with which duplicates with each solution agreed. Blood 3 was controlled with particular care. One sample each was analyzed in ammonia, water, lactic acid, and sodium carbonate solution in the order mentioned, then the entire series was repeated three times in the same order. The possibility of a change in the oxygen content

of the blood, such as might have been conceived had all the ammonia solutions been done together, then all the water solutions, etc., was excluded. The results obtained were, nevertheless, in cc. of gas reduced to 0°, 760 mm., from 2 cc. of blood, the following: ammonia, 0.431, 0.431, 0.435, 0.433, average 0.433; water, 0.430, 0.430, 0.430, 0.431, average 0.430; lactic acid, 0.415, 0.408, 0.409, 0.417, average 0.410; carbonate, 0.409, 0.410, 0.410, average 0.410.

The variations might be explainable on the basis of the previously mentioned oxygen reabsorption if the alkaline solutions were regularly lower, but they are not. All that one can conclude at present is that the maximum, or nearly maximum amount of oxygen is yielded

TABLE VI.

Oxygen Contents of a Series of Bloods Determined after Laking in Alkali, Water, and Acid, Respectively.

Blood No.	Species.	$O_2 + N_2$ content.			
		Ammonia 0.225 millimol per cc. of blood.	Water.	Lactic acid 0.1 millimol per cc. of blood.	Na_2CO_3 0.28 millimol per cc. of blood.
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	Horse ++	17.58	17.70	17.65	
2	Man ++	20.73	20.91	20.95	
3	Dog ++++	21.5	21.5	20.5	20.5
4	Dog ++	21.5	20.9	20.5	
5	Man ++	23.2	23.7	23.6	

++ Two agreeing determinations with each reagent.
++++ Four " " " " "

by blood treated with ferricyanide in water solution; and that markedly increasing the alkalinity greatly reduces the yield. The amounts of ammonia hitherto used, 0.15 to 0.20 millimols per cc. of blood, may in some bloods cause variations of a fraction of a volume per cent of oxygen from the results with water; and similar variations may be caused by the amount of lactic acid (0.1 millimol per cc.) required to free all the CO_2.

5. Comparison of Results of Oxygen Capacity Determinations by Present Method and Haldane Method.

Comparative determinations were carried out in a series of five bloods by our method as described in this paper, with water to lake the blood, and by Haldane's recent modification of his method. The results given in Table VII are each the average of two or more determinations. The results by Haldane's method average 1 volume per cent lower than those by ours. It appears probable that the relatively low results by Haldane's method are due to the difference

TABLE VII.

Oxygen Capacities of a Series of Bloods Determined by the Authors' and by Haldane's Method.

No.	Authors' method.	Haldane method.	
	vol. per cent O_2	*vol. per cent O_2*	*per cent of capacity by authors' method.*
1	21.78	19.80	90.9
2	21.84	20.84	95.4
3	19.37	19.62	101.2
4	19.62	18.75	95.6
5	15.29	14.30	93.6
Average			95.3

in the laking solutions used, rather than to the difference in apparatus employed to measure the oxygen evolved, since when instead of water we used 1 per cent Na_2CO_3 solution (as in the Haldane method) to lake the blood in our own apparatus we encountered similarly low results.

Determination of Carbon Dioxide in Whole Blood and Plasma.

The following changes have been made in the original methods.

1. Transfer of Blood into Apparatus.—The 1 per cent ammonia solution used for washing out the cup of the apparatus[3] before each determination has been dispensed with. Unless kept with especial precautions it gradually absorbs carbon dioxide from the air, and

[3] Van Slyke (1917), p. 354.

consequently must be continually tested. Otherwise a few drops left in the apparatus may carry enough carbon dioxide to make a measurable plus error in the results.

At present we merely rinse the cup out with water before each determination, and then run into it 1 cc. of distilled water. The blood or plasma is run under this layer of water. Even if the latter has an acid reaction it affords an efficient mechanical hindrance to loss of carbon dioxide from the blood in the short time that the latter is in the cup. A drop of octyl alcohol is added, and the blood or plasma, followed by the layer of water, is run into the chamber of the apparatus. The layer of water, thus following the blood, serves to wash down the walls of the cup. In the case of whole blood, some corpuscles settle on the bottom of the cup. These are suspended in the water by stirring them up with the last 0.5 cc. of water after the first 0.5 cc. has passed into the chamber.

After the blood or plasma, water, and octyl alcohol in successive layers have run into the apparatus, leaving only the drop of alcohol in the capillary above the cock, the 0.5 cc. of acid is measured into the cup and run through into the chamber.

2. Lactic Acid Instead of Sulfuric for Decomposing the Blood Bicarbonate.—With plasma, sulfuric acid is entirely convenient, but when sulfuric acid is added to whole blood it causes a heavy protein precipitate, which adds some difficulty to accurate reading and subsequent cleaning of the apparatus. L. L. Van Slyke and Baker (1919) encountered a similar difficulty with casein precipitates in using the apparatus to determine carbon dioxide in milk, and obviated it by substituting lactic acid for sulfuric, the lactic holding the protein in solution instead of precipitating it. We have found that a similar advantage is gained by substituting N lactic acid for N sulfuric in the analysis of whole blood. The hemoglobin forms a clear burgundy solution with a smooth meniscus to read, and after the analysis is completed the apparatus may be cleaned by simply rinsing with water. Lactic acid is made up with sufficient accuracy for the purpose by diluting 1 volume of concentrated acid (specific gravity 1.20) to 10 volumes with water.

3. Absorption of CO_2 in Analysis of Whole Blood.—For absorption of the CO_2 in the mixture of gases obtained after extraction of whole

blood we find 0.5 N NaOH preferable to the 10 per cent KOH recommended previously.[4] The more dilute alkali is of such light specific gravity that it floats on top of the blood, and makes a clear meniscus for reading the final volume. It should be admitted into the chamber with *slight* negative pressure, the mercury in the levelling bulb being held a few cm. below that in the chamber. If it is held much lower a slight amount of air may be extracted from the NaOH solution.

4. Measurement of CO_2 without Removal of Blood Solution from the Measuring Chamber.—When the manipulations are properly performed, it is possible to leave out one step in the analysis; *viz.*, running the solution out of the main chamber into the lower bulb before releasing the vacuum. The vacuum may be released and the gas volume read with all the solution in the chamber, and yet without increased absorption of CO_2, if the following procedure is followed. After extraction of the gas the lower cock is opened, admitting the mercury into the extraction chamber rapidly until the meniscus of the water solution reaches the contracted upper portion of the chamber. At this moment the lower cock is partially closed, and the remainder of the mercury is admitted at a rate sufficiently retarded to *prevent oscillation of the water column* in the calibrated portion of the apparatus, when pressure equilibrium is reached. The pressure is then adjusted by placing the mercury surface in the levelling bulb above the mercury meniscus in the chamber by a height equal to 1/13 that of the water column, in order to balance the latter. After some practice controlled by a centimeter rule, one can estimate this level with the eye to within 2 mm. of mercury, which is sufficiently accurate for many purposes. A more accurate practice, however, is to use the leveling scale described by Stadie (1921) and avoid the possibility of any error whatever in regulating the pressure. When the pressure has been adjusted the lower cock of the apparatus is closed. The gas volume may then be read at leisure.

5. Cleaning Apparatus after Determination of CO_2 in Whole Blood.—When, as in most plasma analyses, the CO_2 is not reabsorbed no washing of the apparatus is necessary before using it for another determination, since the acid solution which wets the walls of the chamber con-

[4] Van Slyke (1917), p. 356.

tains a neglible amount of CO_2. When the CO_2 is reabsorbed by alkali solution, however, as in analysis of whole blood, the solution retains all the absorbed CO_2 and must be completely washed out before another analysis is performed. The washing is conveniently carried out as follows: The upper cock of the apparatus is closed, and the levelling bulb dropped to its lowest level. While the mercury in the apparatus is falling, the 6 cc. cup at the top is filled with water. By this time the apparatus is evacuated and nearly all the water from the cup is let in, washing the entire chamber. The water is ejected, and the rinsing repeated once more, 1 cc. of water being left in the cup to start the next analysis. Washing the apparatus out in this manner consumes less than a minute.

6. Correction for CO_2 Reabsorbed After Release of Vacuum.

While the mercury is rising in the apparatus during release of the vacuum after extraction of CO_2 the layer of water over the mercury surface has an opportunity to reabsorb some of the CO_2 gas. In the original method (Van Slyke, 1917) such reabsorption was guarded against by withdrawing the water solution as completely as possible into the bulb below the lower cock before the vacuum was released. By this expedient the volume of the water layer left on the mercury was reduced to 0.01 to 0.02 cc. Control analyses with standard solutions of Na_2CO_3 gave theoretical results, so that it seemed that the error due to reabsorption had been made negligible.

We have found, however, that although small, it is not negligible. The film of water as it rises in the apparatus reabsorbs 1.5 to 2 per cent of the total amount of CO_2 present. The fact that in the former control analyses 100 per cent instead of 98 to 98.5 per cent of the theoretical amount of CO_2 was obtained from standard Na_2CO_3 solutions was presumably due to the presence of a slight excess of CO_2 absorbed from the laboratory air by the strongly alkaline standard stock solution during the interval between its preparation and the analyses. Such absorption may occur more rapidly than at the time was realized.

Concerning the reabsorption of CO_2 in the apparatus after release of the vacuum, we have ascertained the following.

a. The amount of CO_2 reabsorbed is a constant proportion of the amount present.

b. The reabsorption occurs almost entirely during the last part of the compression, while the gas volume is undergoing reduction from 5 cc. to its final volume.

c. It is independent of the volume of water solution on the surface of the mercury. The absorption apparently is confined so nearly to the surface during the short period involved that depth of layer has relatively slight influence. The volume of water solution may be varied from 0.10 to 2.5 cc. without varying the volume of CO_2 reabsorbed.

d. Variations within wide limits (*e.g.*, 5 and 40 seconds) in the period during which the mercury is allowed to rise have no appreciable influence on the volume of CO_2 reabsorbed.

e. The one factor that may markedly increase the proportion of CO_2 reabsorbed, is the manner in which the meniscus of water over mercury is brought to rest at atmospheric pressure in the measuring tube. If, as the meniscus approaches the narrower upper part of the apparatus, its rise is retarded, and the surface is allowed to come gently to the point of equilibrium with no oscillation, a minimum and constant proportion of CO_2 (1.7 per cent of the total) is reabsorbed. On the other hand, if the final rise is permitted to be violent, and the mercury with the water above it in consequence oscillates several times to a distance of several mm. above and below the point corresponding to atmospheric pressure before it comes to rest, 0.01 to 0.02 cc. of CO_2 may be reabsorbed in the process.

Certain of the above points are demonstrated by the following experiments.

Analyses of Standard Na_2CO_3 Solutions.—Na_2CO_3 was prepared from Merck's "Reagent" $NaHCO_3$ by heating for several hours in an oven at 250°C. The Na_2CO_3 thus prepared was kept in bottles with glass stoppers that had been rendered tight by regrinding and greasing.

A 0.03 molecular solution of Na_2CO_3 (3.180 gm. Na_2CO_3 per liter) was prepared by weight, and checked by titrating against 0.1 N HCl. 20 cc. neutralized to methyl orange 11.97 and 12.04 cc. of 0.1 N HCl, after subtraction of 0.10 cc. of 0.1 N HCl required to give the chosen end-point in a control water solution. Calculated 12.00 cc.

The CO_2 content was determined with an apparatus of the usual size (50 cc. chamber, 1 cc. measuring scale), but designed like the micro-apparatus described by Van Slyke (1917, p. 363), so that there was no chance for reabsorption of CO_2 during release of the vacuum. Found, 67.4, 67.0, and 67.2 volumes per cent CO_2. Calculated 67.2. Found = 100.0 per cent of calculated.

The CO_2 content of this solution was redetermined on 1 cc. samples with the ordinary macro-apparatus (Van Slyke, 1917, p. 349) with a single extraction. Found, 65.5, 66.2, 65.9, 66.2, 66.4, average 66.0 volumes per cent. Found = 98.2 per cent of calculated.

A 0.015 M solution was prepared by dilution of the above 0.03 M solution with CO_2-free water, and the CO_2 was determined by a single extraction in the same apparatus. Found, 33.1, 33.1, 33.1, and 33.1 volumes per cent of CO_2. Calculated 33.6. Found = 98.5 per cent of calculated.

Direct Determination of Reabsorbed CO_2.—The determinations were carried out in the fine bore macro-type apparatus (Fig. 1) and were performed in the usual manner up to the point at which the vacuum is released. The mercury was allowed to rise to the desired point. At this point the outlet capillary, *a*, was connected with a suction pump. Then the supernatant atmosphere was freed of CO_2 by applying suction, letting in air, and applying suction again, by revolving the upper cock. This operation was performed in a few seconds, during which the solution in the chamber was kept quiet, in order to avoid reextracting the CO_2 that it held in solution. The air was then driven out through the cup at the top, the solution being driven after it into the cup, where it remained for a few seconds while the mercury seal of outlet, *a*, was reestablished. The solution was then readmitted into the chamber, and its CO_2 was extracted and measured. The measurements of the small volumes of gas were made after reducing the pressure by 500 mm. of mercury, as described in this paper, and the purity of the CO_2 was controlled by absorption with NaOH. The results are given in Table VIII.

From the preceding analyses of standard carbonate solutions we have shown that one extraction by the usual technique yields 98.3 to 98.5 per cent of the calculated CO_2 volume, while from the analysis, in Table VIII, of the untrapped solution remaining in the chamber

we have recovered 1.5 to 2.2, on the average 1.7 per cent of CO_2 reabsorbed during the rise of the mercury to the point of establishment of atmospheric pressure (Determinations 9 to 15). It is therefore demonstrated in two ways that reabsorption diminishes by a quite constant proportion, *viz.* 1.7 per cent, the amount of CO_2 obtained by the single extraction method used as originally described

TABLE VIII.

Determination of Reabsorbed CO_2.

No.	*a* CO_2 in Na_2CO_3 solution analyzed.	*b* Volume of free space left in chamber over Hg when CO_2 was replaced by air.	*c* Volume of untrapped solution left in chamber.	*d* CO_2 obtained from 2nd extraction of untrapped solution.	*e* CO_2 estimated left in untrapped solution after 1st extraction.*	*f* CO_2 reabsorbed after 1st extraction.	
						$c - d$	$100 \frac{c-d}{a}$
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent of total*
1	0.672	50	0.17	0.002	0.002	0.000	0.0
2	0.672	50	0.25	0.004	0.003	0.001	0.1
3	0.672	25	0.29	0.004	0.004	0.000	0.0
4	0.672	5	0.20	0.005	0.003	0.002	0.3
5	0.672	5	2.50	0.033	0.030	0.003	0.4
6	0.672	2	0.20	0.007	0.002	0.005	0.7
7	0.672	1	0.16	0.012	0.002	0.010	1.5
8	0.672	1	0.10	0.012	0.001	0.011	1.6
9	0.672	0.78	0.18	0.014	0.003	0.011	1.6
10	0.672	0.78	0.20	0.014	0.003	0.011	1.6
11	0.672	0.74	0.18	0.013	0.003	0.010	1.5
12	0.336	0.40	0.10	0.007	0.001	0.006	1.8
13	0.336	0.40	0.20	0.009	0.0016	0.0084	2.2
14	0.336	0.40	2.50	0.024	0.018	0.006	1.8
15	0.336	0.40	2.50	0.023	0.018	0.005	1.5

* Calculated as $a\ c \frac{\alpha'_{CO_2}}{A + (\alpha'_{CO_2} - 1)\,S}$

by Van Slyke. The constancy of the correction justifies its introduction into the calculations by multiplying the theoretically established factors by $\frac{1}{0.983} = 1.017$. This we have done in Table XIII.

Alternatives to using this correction factor are: (1) To reextract the untrapped water solution after each analysis, as described in the

experiments reported in Table VIII, and (2) to use the micro-apparatus (Van Slyke, 1917) in the original or an enlarged form. In such an apparatus the water solution may be entirely separated from the gas phase before the vacuum is released, and reabsorption of CO_2 therefore made impossible.

We have found, however, that with care to prevent oscillation of the mercury-water layer after atmospheric pressure is reached, such constant results are obtained with the simpler, usual apparatus and technique (see Table I), that we prefer to continue its use, merely introducing the factor 1.017 to correct for the reabsorption. This correction involves a change of about 1 volume per cent in the results of an ordinary blood or plasma CO_2 determination.

7. *Calculation of CO_2 Results.*—In deriving the formula by which results were calculated in the original paper (Van Slyke, 1917, p. 358) the factor $\frac{S}{50}\alpha'_{CO_2}$ was used to calculate the fraction of the gas remaining dissolved in the water when equilibrium was reached in extraction. (S = cc. of water solution, 50 = cc. volume of extraction chamber, α'_{CO_2} = cc. of CO_2, measured at the prevailing temperature, dissolved by 1 cc. of water in equilibrium with CO_2 gas at 760 mm. tension.) This factor represents an approximation that is entirely exact only when $\alpha'_{CO_2} = 1$, which occurs at 18°. The deviation between the above approximate solubility correction factor and the exact factor increases, (1) as α'_{CO_2} becomes greater or less than 1, and (2) as the ratio $\frac{S}{50}$ increases. For the conditions under which the approximate factor was used, however, (temperature range 15 – 30°, $\frac{S}{50} = 0.05$) the error introduced does not exceed 1 part per 1,000. Consequently the numerical factors arrived at by the original approximate equation do not require correction for the sake of accuracy.

It seems, however, desirable to present the exact general equation which expresses the relationships between gas and liquid phases under conditions such as those prevailing in the apparatus.

V_t = volume of CO_2 obtained by one extraction and measured at atmospheric conditions of t° temperature and B mm. barometric pressure.

$V_{0°,760}$ = total volume of CO_2, reduced to 0°, 760 mm., in the solution analyzed.

S = volume of water solution in apparatus.

A = volume ot chamber occupied by gas and solution during extraction (50 cc. in our apparatus).

$A - S$ = volume of gas phase during extraction.

T = absolute temperature, $= t + 273$.

α_{CO_2} = solubility coefficient of CO_2 in water, the cc. of CO_2 measured at 0°, 760 mm., dissolved by 1 cc. of water in equilibrium with CO_2 under 760 mm. tension.

$\alpha'_{CO_2} = \alpha_{CO_2} \times \frac{T}{273}$ = distribution coefficient of CO_2 between gas and water = cc. of CO_2 measured at $t°$, B mm. dissolved by 1 cc. of water in equilibrium with pure CO_2 at $t°$, B mm. (α'_{CO_2} was referred to as α_{CO_2} in the original paper.)

w = vapor tension of water.

p = partial pressure of CO_2 in apparatus when equilibrium is reached in the extraction.

x = volume of CO_2 gas, measured at 0°, 760 mm., held in solution when equilibrium is reached.

The total CO_2 content of the solution analyzed is obtained by reducing the volume of CO_2 extracted to standard conditions by multiplication with the usual factor $\frac{(B - w)\ 273}{760\ T}$, and adding to the volume thus corrected the volume x of CO_2 remaining in solution. Thus:

(1) $$V_{0°,760} = V_t \frac{B - w}{760} \times \frac{273}{T} + x$$

Since the volume of gas dissolved is proportional to its partial pressure, solubility, and the volume of the solvent, we have

(2) $$x = \frac{p}{760} S\alpha_{CO_2}$$

Since pressure varies inversely as gas volume

(3) $$\frac{p}{B - w} = \frac{V}{A - S}, \text{ or}$$

(4) $$p = (B - w)\frac{V}{A - S}$$

Substituting this value for p in (2), and the value thereby found for x in (1), we have

(5) $$V_{0°,760} = V_t \frac{B - w}{760} \times \frac{273}{T} + V \frac{B - w}{760} \times \frac{S\alpha_{CO_2}}{A - S}, \text{ or}$$

(6) $$V_{0°,760} = V_t \frac{B - w}{760}\left(\frac{273}{T} + \frac{S\alpha_{CO_2}}{A - S}\right)$$

Since $\alpha_{CO_2} = \alpha'_{CO_2} \times \frac{273}{T}$, equation (6) may be expressed as

(7) $$V_{0°,760} = V_t \frac{B - w}{760} \times \frac{273}{T}\left(1 + \frac{S}{A - S}\alpha'_{CO_2}\right), \text{ or}$$

(8) $$V_{0°,760} = V_t \underbrace{\frac{B - w}{760\ (1 + 0.00367\ t)}}_{\text{Factor correcting for atmospheric pressure and temperature.}} \underbrace{\left(1 + \frac{S}{A - S}\alpha'_{CO_2}\right)}_{\text{Factor correcting for unextracted } CO_2.}$$

In the approximate equation used in the original paper the factor correcting for unextracted CO_2 was $\frac{1}{1 - \frac{S}{A}\alpha_{CO_2}}$. When $\alpha'_{CO_2} = 1$ (at 18°) this becomes identical with $1 + \frac{S}{A - S}\alpha'_{CO_2}$, both factors then reducing to $\frac{A}{A - S}$.

The values of the combined factor $\frac{B - w}{760\ (1 + 0.00367\ t)}\left(1 + \frac{S}{A - S}\alpha'_{CO_2}\right)$, multiplied by 1.017 to correct for reabsorption of CO_2 during release of the vacuum, are given in Table XIII at the end of this paper for the calculation of results. Expressing $\frac{B - w}{760\ (1 + 0.00367\ t)}$ as f, we have given its values for B = 760, to

be multiplied by $\frac{B}{760}$ for other barometric pressures. Because the vapor tension, w, is not quite the same fraction of $B - w$ when B is other than 760, this usage is not absolutely exact. At a barometric pressure $B = 740$ it introduces an error of plus 0.1 per cent, at 700 mm., an error of plus 0.4 per cent. For barometric pressures outside the range 740 to 780 mm., therefore, one must use the customary tables for values of $\frac{B - w}{760\ (1 + 0.00367\ t)}$ in order to avoid errors exceeding 1 part per 1,000. For work at ordinary altitudes, however, the factors as given in Table XIII are sufficiently exact.

Determination of Carbon Monoxide.

In the method of Van Slyke and Salvesen (1919) it appears slightly more accurate to use 1.36 volumes per cent as the correction for nitrogen gas rather than the 1.2 volumes per cent correction found by the above authors. Still more accurate results may be obtainable by absorption of the CO with ammoniacal, cuprous chloride solution as recently described by O'Brien and Parker (1921).

In the determination of carbon monoxide it is advisable to use the finer bore apparatus and to magnify the gas volumes by reducing the pressure as described at the beginning of this paper, since the amounts of gas measured are likely to be much smaller than those measured when carbon dioxide or oxygen is determined.

The blood should be trapped in the lower bulb of the apparatus before releasing the vacuum, as it is undesirable to mix blood with pyrogallol and cuprous chloride.

Determination of Methemoglobin.

In the determination of methemoglobin (Stadie, 1920) the total blood pigment is determined colorimetrically, and the methemoglobin is estimated by subtracting from the total pigment the oxyhemoglobin. The latter is determined by the oxygen capacity method. The oxygen capacity portion of the methemoglobin determination therefore requires revision in its method of calculation, as outlined above. Instead of utilizing Table I of Stadie's paper the oxygen

capacity of the blood is determined and calculated as described above, to make complete allowance for the nitrogen gas content of the blood. The volume per cent of oxygen is then multiplied by 0.746, the number of gm. of hemoglobin that combines with 1 cc. of oxygen. The result is the number of gm. of hemoglobin per 100 cc. of blood.

The change in the mode of calculation affords sufficient increase in accuracy to be justified, but the error involved in the former method is too slight to invalidate any results that have been obtained by it. The absolute error is 0.5 volume per cent in oxygen capacity (equivalent to 0.37 gm. of hemoglobin per 100 cc. of blood) or about 2.5 per

TABLE IX.

Methemoglobin Calculation.

	Hemoglobin per 100 cc. of blood.	
	Calculated as in original paper (Stadie, 1920).	Calculated as described in this paper.
	gm.	*gm.*
Hemoglobin strength of standard blood calculated from oxygen capacity	15.0	14.6
Total blood pigment colorimetrically determined = $\frac{1}{1}\frac{0}{2}$ of standard	12.5	12.2
Hemoglobin determined by oxygen capacity	10.0	9.6
Methemoglobin by difference	2.5	2.6

cent of the amount normally present. In the methemoglobin calculation this error is partly neutralized by the fact that the same percentage error is introduced into the colorimetric estimation of total pigment by making the standard methemoglobin solution from blood in which the hemoglobin had been estimated by the same oxygen capacity method. For example, in Table IX the model calculation given on page 240 of Stadie's paper (1920) is reproduced together with the calculation as corrected in this paper. It is seen that the change in methemoglobin calculated is but 0.1 gm. per 100 cc. of blood.

Determination of All the Gases in One Blood Sample.

Both carbon dioxide and oxygen, as well as the residual nitrogen, may be determined in one sample of blood with but little more expenditure of time and effort than is required to determine any one of the gases alone. For liberation of the gases both acid and ferricyanide are added to the blood in the apparatus, and all the gases are extracted and measured together. The CO_2 is then absorbed by dilute alkali, leaving O_2 and N_2. The O_2 may then be determined by absorption with pyrogallol, or estimated by subtracting the average N_2 content of blood from the sum of $O_2 + N_2$.

The essential point in determining the gases together was found to lie in the use of proper amounts of the reagents employed to set free the carbon dioxide and oxygen, particularly in the use of a minimum amount of acid. If potassium ferricyanide and a large excess of acid are added to blood, both oxygen and carbon dioxide are quantitatively freed, but the ferricyanide forms such a heavy precipitate with the blood proteins that it is inconvenient, although possible, to handle the resulting suspension in the apparatus. The precipitation of the proteins by ferricyanide occurs, however, only when the reaction is strongly acid. If but the minimum amount of lactic acid necessary to free the CO_2 with certainty is added, the ferricyanide-protein precipitate formed is so small in amount and so finely divided that it does not interfere with the determination. Furthermore, the acid can be trapped in the lower bulb of the apparatus before the blood is admitted, and can then be mixed with the blood after the apparatus has been evacuated. The blood is then in the lower part of the evacuation chamber, and such precipitate as does form does not touch the measuring tube at the top, which remains clean and clear. The precipitate, unlike the black deposit formed by action of ferricyanide on the mercury in the ammoniacal solution used in the original oxygen determination, is instantly soluble in 0.1 N alkali. Consequently any particles that adhere to the walls of the apparatus are removed as a rule by a single washing with dilute alkali solution.

The minimum amount of acid that will entirely free the CO_2 of whole blood for extraction under the conditions of our analyses was determined by preliminary experiments, some of which are recorded in Table X. The amount needed was found to be 1.0 cc.

of 0.1 N lactic acid for 1 cc. of whole blood. Half as much fails to set the CO_2 completely free.

The minimum amount of ferricyanide required in the presence of acid is the same as in ammoniacal solution; *viz.*, 5 mg. of potassium ferricyanide per 1 cc. of normal blood (Table II). In avoiding protein precipitation, however, the amount of ferricyanide present appears to be of much less importance than the acidity of the mixture; if the acidity is too high, the minimum necessary amount of ferricyanide causes an inconvenient precipitate, while if the acidity is not too high an excess of ferricyanide may be used without such inconvenience. We consequently employ 10 mg. of ferricyanide per cc. of blood, twice the necessary amount.

TABLE X.

Amounts of Acid Required to Free CO_2 in Human Whole Blood.

Lactic acid added to 1 cc. of blood.	CO_2 found in blood.
millimols	*vol. per cent*
0.05	41.5
0.10	52.8
0.10	53.3
0.20	52.8

The analysis is performed as follows:

Apparatus Used.—In order to obtain with 1 cc. of blood results for carbon dioxide and oxygen accurate to within 1 per cent of the amounts measured, it is desirable to use the fine bore type of apparatus described at the beginning of this paper, although, with care, results sufficiently accurate for many purposes may be obtained with the ordinary apparatus.

Details of Determination.—First 2 cc. of 0.05 N lactic acid are admitted into the chamber of the apparatus. The acid is freed of air by evacuation and shaking in the usual manner. The air-free acid is then trapped in the bulb below the lower cock of the apparatus, and 1.9 cc. of water, with a drop of octyl alcohol, are similarly introduced and freed of air. The blood is now stirred and a sample drawn into an Ostwald pipette calibrated between two marks

to deliver 1 cc. About 1.5 cc. of the air-free water in the gas analysis apparatus are run into the cup at the top, and the blood sample is at once run beneath it. We usually slightly open the cock below the cup while the pipette is draining, so that most of the blood flows directly into the chamber of the apparatus, the layer in the cup at no time being more than 2 to 3 mm. deep. The layer of water, even though it be somewhat acidified, prevents, because of the relatively slow rate of diffusion through it, the loss of CO_2 from the blood. All the blood, followed by the water layer above it, is now admitted into the chamber of the apparatus. When about half the water layer has been run in, the half remaining in the cup is stirred with a rod in order to get into suspension a few corpuscles that have lodged on the bottom of the cup. 0.05 cc. of solution containing 20 gm. of potassium ferricyanide per 100 cc. is added to this last portion of water, which is then admitted into the chamber. The 0.05 cc. of ferricyanide solution may be conveniently measured as 1 drop from a pipette, which has been found by trial to deliver thus 0.05 to 0.06 cc. of the solution.

The chamber is evacuated until the mercury has fallen to the 50 cc. mark. The 2 cc. of 0.05 N lactic acid trapped in the lower bulb of the apparatus are now admitted and mixed with the rest of the solution. At this moment a small amount of brown precipitate forms, but not enough to interfere with the subsequent manipulations. The oxygen and carbon dioxide (and carbon monoxide if present) are extracted by whirling the solution about the wall of the evacuated chamber until, when the vacuum is released and the gas measured, no increase in volume is observed. Complete extraction is usually attained in 1 minute when the apparatus is shaken by hand. When the mechanical shaker devised by Stadie (1921) has been used, 2 minutes shaking after the first evacuation, followed by 30 seconds shaking for the check evacuations, has been our usual routine. As a rule no increase occurs after the first evacuation. Increase after the second indicates a leak in the apparatus, due usually to improper lubrication of the upper cock.

When extraction of the gases is complete the water solution is trapped below the lower cock, as in the original CO_2 determination, and the gases are measured at atmospheric pressure.

TABLE XI.

Comparison of Carbon Dioxide and Oxygen Contents of Normal Venous Blood Determined Separately by Former Methods, and Together by the Present Combined Method, Respectively.

Blood kept under paraffin oil. All determinations made within 3 hours after blood was drawn.

Determination.	Method.	Volume of blood for analysis.	CO_2	$O_2 + N_2$	O_2	Potassium ferricyanide used.
		cc.	*vol. per cent*	*vol. per cent*	*vol. per cent**	*mg.*
O_2 alone.	Van Slyke, 1918.	2		21.1	19.6	40
O_2 "	" " 1918.	2		20.9	19.4	40
CO_2 "	" " 1917.	1	65.1			
CO_2 "	" " 1917.	1	65.5			
CO_2 "	" " 1917.	2	65.0			
$O_2 + CO_2$.	Present.	2	64.4	20.5	19.0	10
$O_2 + CO_2$.	"	2	65.0	20.9	19.4	10
$O_2 + CO_2$.	"	2	63.5	20.9	19.4	15
$O_2 + CO_2$.	"	2	64.4	20.9	19.4	30
$O_2 + CO_2$.	"	2	64.2	20.9	19.4	70

* The N_2 was determined and found to be 1.5 volumes per cent. It is deducted and subtracted from the $O_2 + N_2$ to estimate the O_2.

TABLE XII.

Comparison of Carbon Dioxide and Oxygen Contents Determined Separately by Former Methods, and Together by the Present Combined Method.

All determinations made on 1 cc. samples in a fine bore apparatus.

	Oxygen.		CO_2	
	Method.			
	Former.	Present.	Former.	Present.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Blood aerated with alveolar air	18.1	17.9	58.7	58.2
" diluted with saline	9.4	8.8	38.7	39.5
Venous blood about 60 per cent saturated with O_2.	13.6	13.9	35.8	36.4
0.02625 M Na_2CO_3 (calculated 58.8 volumes per cent CO_2)			58.7	59.1

TABLE XIII.

Factors for Calculation.

Temperature.	$f = \frac{B}{760\ (1 + 0.00367\ t) - w}$ factor by which gas measured moist at $t°$, B mm., is reduced to 0°, 760 mm.*	α'_{CO_2}	Air, † measured at room temperature and pressure, dissolved by		$1.017\ f\ (1 + \frac{S}{50 - S}\ \alpha'_{CO_2})$, factor by which the volume of CO_2 obtained after 1 extraction is multiplied in order to obtain the volume of CO_2 reduced to 0°, 760 mm., contained in the solution analyzed.	
			2.5 cc. H_2O.	5 cc. H_2O.	S = 2.5 cc.	S = 5.0 cc.
°C.			cc.	cc.		
15	$0.932 \times \frac{B}{760}$	1.075	0.052	0.105	$1.002 \times \frac{B}{760}$	$1.061 \times \frac{B}{760}$
16	0.928 "	1.043	0.051	0.101	0.995 "	1.053 "
17	0.924 "	1.015	0.050	0.100	0.989 "	1.046 "
18	0.919 "	0.989	0.049	0.098	0.983 "	1.038 "
19	0.915 "	0.966	0.048	0.096	0.978 "	1.030 "
20	0.910 "	0.942	0.047	0.095	0.972 "	1.022 "
21	0.906 "	0.919	0.046	0.093	0.966 "	1.015 "
22	0.901 "	0.896	0.045	0.091	0.960 "	1.008 "
23	0.897 "	0.873	0.045	0.090	0.954 "	1.001 "
24	0.892 "	0.850	0.044	0.088	0.948 "	0.993 "
25	0.888 "	0.828	0.043	0.086	0.942 "	0.986 "
26	0.883 "	0.808	0.042	0.084	0.936 "	0.978 "
27	0.878 "	0.789	0.041	0.083	0.931 "	0.971 "
28	0.873 "	0.772	0.040	0.081	0.924 "	0.964 "
29	0.868 "	0.755	0.040	0.080	0.918 "	0.957 "
30	0.863 "	0.738	0.039	0.078	0.912 "	0.950 "

* To calculate O_2 or hemoglobin when $O_2 + N_2$ volume is measured, multiply gas volume by f, to reduce to 0°, 760 mm., and by such factor as is necessary (100 when 1 cc. of blood is used, 50 when 2 cc. are used) to bring results to volume per cent basis. Then for

a. O_2 content, subtract..... 1.36 vol. per cent N_2

b. O_2 bound by hemoglobin in venous blood, subtract..... 1.5 " " " N_2 + dissolved O_2

c. O_2 bound by hemoglobin in arterial blood, subtract..... 1.7 " " " " " " "

TABLE XIII—*Concluded.*

d. O_2 bound by hemoglobin in blood saturated with air at 20°, subtract..... 2.1 vol. per cent N_2+dissolved O_2.

Per cent of normal hemoglobin (Haldane scale) $= \frac{100\,d}{18.5} = 5.41\,d$.

Grams of hemoglobin per 100 cc. of blood $= 0.746\,d$.

Per cent of total hemoglobin saturated with $O_2 = \frac{100\,b}{d}$ or $\frac{100\,c}{d}$.

Volumes per cent O_2 unsaturation $= d - c$ or $d - b$.

b and *c* may be determined with slightly greater accuracy with the aid of Table IV. The values for *f* given in the second column are for barometric readings corrected for temperature (see remarks on p. 31). The values for α'_{CO_2} are obtained by multiplying by $1 + 0.00367\,t$ the values for α_{CO_2} given by Bohr and Bock (1891).

† The dissolved air is given as measured at room temperature. It is subtracted from the air + CO_2 volume, measured after one extraction of plasma or aqueous carbonate solution, in order to obtain the CO_2, which is then multiplied by $1.017\,f\left(1 + \frac{S}{50 - S}\,\alpha'_{CO_2}\right)$ in order to obtain the total volumes per cent of CO_2 in the solution analyzed. When whole blood is analyzed, the air correction cannot be used, because of the O_2 present, and the CO_2 must be determined by absorption with NaOH solution. The volume of gas absorbed is then multiplied by the above factor.

The factor 1.017, being empirical (see p. 554), may vary slightly for different apparatus.

The gases measured are CO_2, O_2, N_2 and sometimes CO. The CO_2 is absorbed by running 0.5 N NaOH from the cup down the side of the measuring tube until no further shrinkage of the gas volume occurs; about 0.5 cc. of the hydroxide solution suffices.

As the volume, S, of solution in the apparatus is twice as great as in the method as originally described, the factors used for calculation of carbon dioxide are different. For the present conditions, with 5 cc. of total water solution extracted in the apparatus, the factors are given in Table XIII.

If no CO is present, the residual mixture left after absorption of CO_2 may be measured as O_2 + N_2, and from the O_2 + N_2 content reduced to 0°, 760 mm. (by Table XIII), the average N_2 content of

blood, *viz.*, 1.36 volumes per cent, may be subtracted in order to estimate the oxygen; or the oxygen may be measured directly by absorption, as described by Van Slyke and Salvesen. In the latter case about 0.5 cc. of pyrogallol solution is run in from the cup, and is followed by a little water to clean the tube and give a clear meniscus for reading.

If carbon monoxide is present, the oxygen must, of course, be absorbed by pyrogallol. The residual gas is the CO + 1.36 volumes per cent of N_2. The CO is calculated by reducing the mixture of CO and N_2 to 0°, 760 mm. (Table XIII), and subtracting 1.36 from the result in volumes per cent, or the CO may be absorbed by cuprous chloride (O'Brien and Parker, 1921).

The results of some determinations are given in Table XI and XII.

Examples of Calculations.

1. Total Oxygen Content of Venous or Arterial Blood.

a. From O_2 and N_2 Measured Together.

	Blood sample	2.00	cc.
	$O_2 + N_2$ measured	0.405	" at 25°, 750 mm.
$0.405 \times 0.888^* \times \frac{750}{760}$	$= O_2 + N_2$	0.3534	" " 0°, 760 "
0.3534×50	$= O_2 + N_2$ per 100 cc. blood	17.73	" " 0°, 760 "
	N_2 subtracted	1.36	" " 0°, 760 "
	O_2 by difference	16.37	" " 0°, 760 "

b. From O_2 Determined by Absorption with Pyrogallol.

	Blood sample	2.00	cc.
	$O_2 + N_2$ measured	0.405	" at 25°, 750 mm.
	N_2 after absorption of O_2	0.031	" " 25°, 750 "
	O_2 by difference	0.374	" " 25°, 750 "
0.374×50	$= O_2$ per 100 cc. blood	18.70	" " 25°, 750 "
$18.70 \times 0.888^* \times \frac{750}{760}$	$= O_2$ " 100 " "	16.37	" " 0°, 760 "

2. Oxygen Combined with Hemoglobin in Venous or Arterial Blood as Drawn.

a. Venous blood.

Total O_2, calculated as above	16.37	cc. at 0°, 760 mm.
O_2 in physical solution	0.10†	" " 0°, 760 "
O_2 combined with hemoglobin	16.27	" " 0°, 760 "

* Factor from second column of Table XIII.

† These figures are approximate and are the average for arterial and venous bloods. For the accurate values of oxygen in physical solution, see Table III.

b. Arterial blood.

Total O_2 calculated as above	16.37	cc. at	0°, 760 mm.
O_2 in physical solution	0.32†	" "	0°, 760 "
O_2 combined with hemoglobin	16.05	" "	0°, 760 "

3. Oxygen Combined with Hemoglobin in Blood Saturated with Air at Room Temperature (Oxygen Capacity).

a. From O_2 and N_2 Measured Together.

Blood sample	2.00	cc.	
O_2 and N_2 measured	0.495	"	at 20°, 767 mm.
$0.495 \times 0.910^* \times \frac{767}{760} = O_2 + N_2$	0.455	" "	0°, 760 "
$50 \times 0.455 = O_2 + N_2$ per 100 cc. blood	22.75	" "	0°, 760 "
Physically dissolved $O_2 + N_2$ per 100 cc. blood	2.10	" "	0°, 760 "
Combined O_2 per 100 cc. blood	20.65	" "	0°, 760 "

b. From O_2 Determined by Absorption with Pyrogallol.

Blood sample	2.00	cc.	
$O_2 + N_2$ measured	0.495	"	at 20°, 767 mm.
N_2 after absorption of O_2	0.033	" "	20°, 767 "
O_2 by difference	0.462	" "	20°, 767 "
$0.462 \times 0.910^* \times \frac{767}{760} \times 50 =$ Total O_2 per 100 cc. blood	21.22	cc. at	0°, 760 mm.
Physically dissolved O_2 per 100 cc. blood	0.58	" "	0°, 760 "
Combined O_2 per 100 cc. blood	20.64	" "	0°, 760 "

4. Calculation of Hemoglobin Content from Oxygen Capacity.

Combined O_2 per 100 cc. blood, calculated as above, 20.64 cc. at 0°, 760 mm.

20.64×0.746 = hemoglobin per 100 cc. blood ..... = 15.40 gm. hemoglobin.

$\frac{20.64}{18.5} \times 100$ = per cent of Haldane's average normal hemoglobin ..... = 112 per cent.

5. Calculation of Oxygen Unsaturation of Blood.

Oxygen combined with hemoglobin per 100 cc. of blood after saturation of blood with air at room temperature (oxygen capacity) (Calculation 3, *b*)	20.64	cc. at	0°, 760 mm.
Oxygen combined with hemoglobin of blood as drawn (calculation 2, *a*)	16.27	" "	0°, 760 "
Oxygen unsaturation	4.37	" "	0°, 760 "
Percentage unsaturation $= 100 \times \frac{4.37}{20.64}$	21.2	per cent.	

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* Factor from second column of Table XIII.

† These figures are approximate and are the average for arterial and venous bloods. For the accurate values of oxygen in physical solution, see Table III.

6. Calculation of Total Gas Content from Analysis of 1 Cc. of Blood as Described in This Paper.

Blood sample	1.00	cc.
a. $CO_2 + O_2 + N_2$ measured	0.845	" at 25°, 757 mm.
b. $O_2 + N_2$ measured after CO_2 absorption	0.248	" " 25°, 757 "
c. N_2 " " O_2 "	0.017	" " 25°, 757 "
CO_2 by difference $(a - b)$	0.597	" " 25°, 757 "
O_2 " " $(b - c)$	0.231	" " 25°, 757 "

$0.597 \times 0.986‡ \times \frac{757}{760} \times 100 =$ CO_2 per 100 cc. blood .... 58.6 " " 0°, 760 "

$0.231 \times 0.888 \times \frac{757}{760} \times 100 =$ O_2 " 100 " " .... 20.4 " " 0°, 760 "

$0.017 \times 0.888 \times \frac{757}{760} \times 100 =$ N_2 " 100 " " .... 1.5 " " 0°, 760 "

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‡ Factor from last column of Table XIII.

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A MECHANICAL SHAKER AND OTHER DEVICES FOR USE WITH THE VAN SLYKE BLOOD GAS APPARATUS.

By WILLIAM C. STADIE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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When many determinations of the blood gases by the Van Slyke method are made from day to day the shaking of the apparatus by hand becomes laborious, and it appears worth while to describe a mechanical shaker which has been used successfully for over a year. In addition several other simple devices are described which have saved time and labor.

Mechanical Shaker.—The construction is made evident by reference to Fig. 1. The pulley is rotated by a small motor not shown in the diagram. The speed is regulated by a lamp and rheostat inserted in series. The shaking given to the apparatus by this device is rapid, efficient, and almost noiseless. There is little danger of breaking the apparatus. We have used two of these shakers for more than a year without such accident.

By means of a switch attached to an interval time clock, the current to the motor may be automatically shut off after the desired interval of shaking. The latter is usually 1 minute for a CO_2 determination in plasma, 2 minutes for either CO_2 or O_2 in whole blood.

The small rubber tube connected with the outlet of the upper stopcock serves, after the analysis has been completed, to carry the expelled solutions to a waste bottle. A few drops of mercury are usually driven out with each portion of waste solution. The mercury thus lost from the apparatus collects in the bottom of the waste bottle, from which it is from time to time recovered, washed, strained through chamois skin, and restored to the levelling bulb.

The bottle which serves to collect the waste residue may be conveniently placed in a sink under an aspirator suction pump, the water from which washes the mercury. The suction may also be used conveniently to draw out waste fluids from the cup of the apparatus.

Levelling Scale.—When, as is usually our practice in oxygen determinations, the reaction mixture is left in the main chamber of the apparatus while the gas is measured, the surface of the mercury

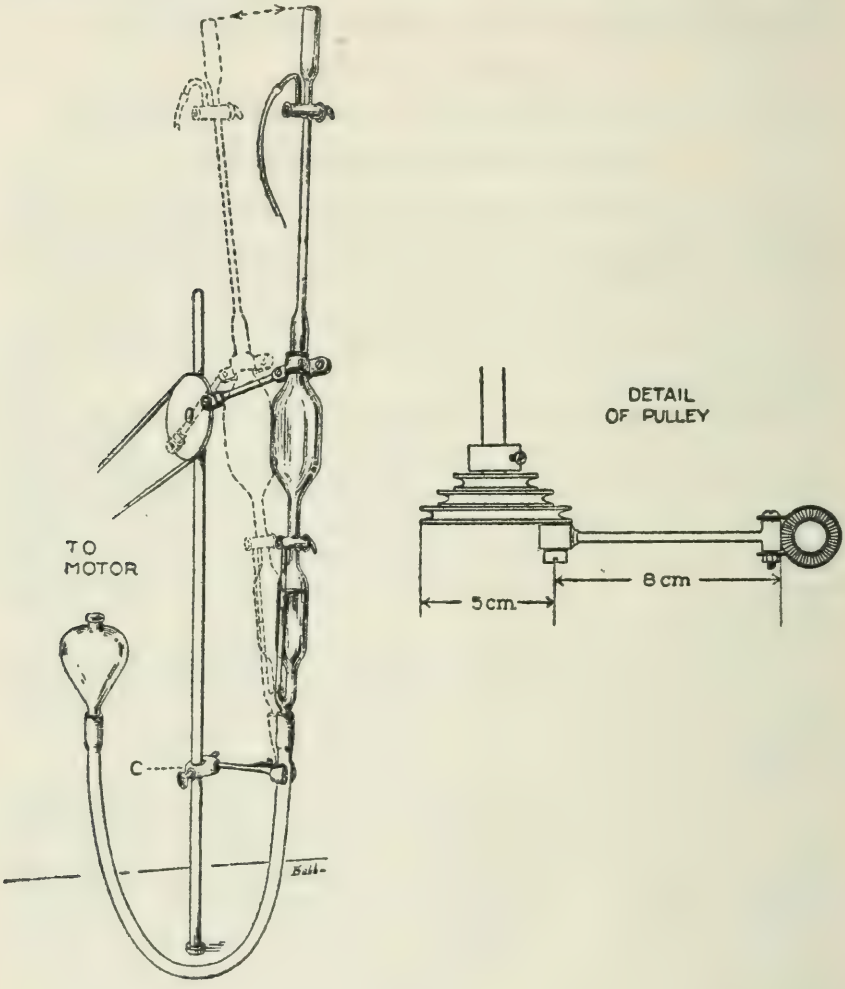

FIG. 1.

in the levelling bulb must be raised sufficiently above that in the apparatus to balance the column of water solution in the latter. For accurate pressure adjustment under these conditions it is desirable to use an empirically calibrated scale, which may be attached to some convenient place on or near the stand holding the apparatus.

The scale is calibrated by placing in the chamber of the gas apparatus an amount of water equal to the final volume of the reaction mixture to be used (*e.g.*, 8 cc. for oxygen analysis of 2 cc. of blood). Then, with the upper cock of the apparatus open, to establish atmospheric pressure within, one marks on the scale the levels at which

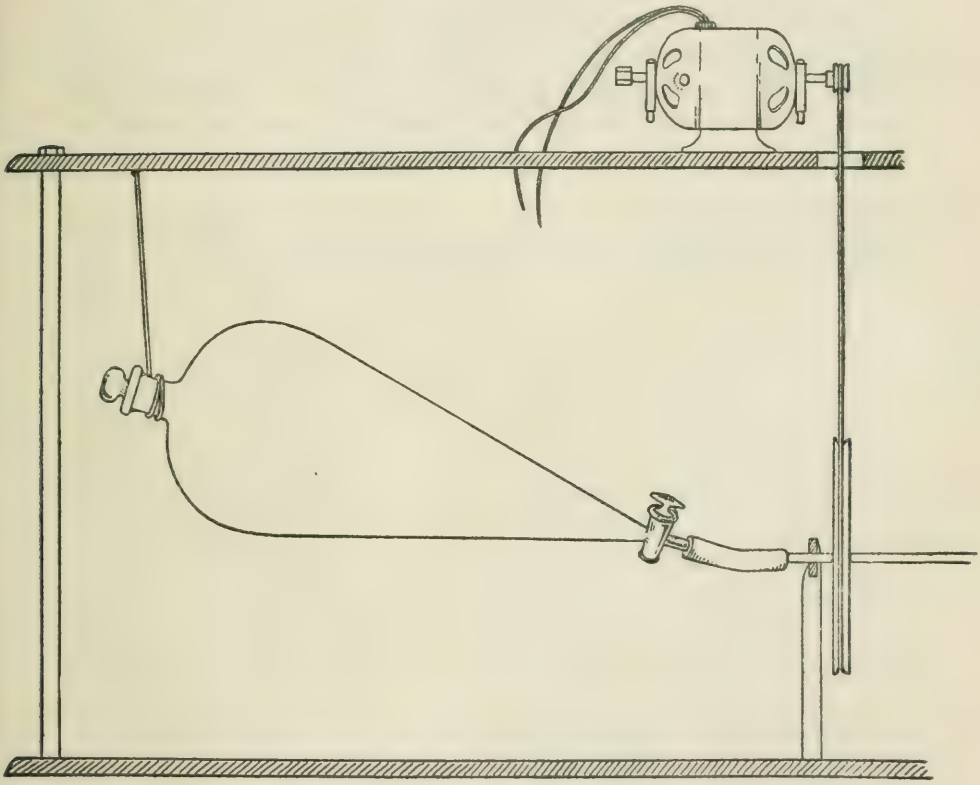

FIG. 2.

the surface of the mercury in the levelling bulb is held when 0.10 0.20, 0.30, etc., cc. of gas are present in the apparatus. In subsequent analyses the levelling bulb is adjusted by comparison with the scale thus prepared. For particularly careful adjustment, the levelling bulb may have an upright side arm of 6 or 8 mm. diameter sealed into

it; the mercury surface in the arm can be levelled against the scale somewhat more readily than the broader surface in the bulb itself.[1]

Tonometer Rotator.—When a separatory funnel is used as a tonometer for saturation of blood with gas, the funnel may be conveniently rotated by the device shown in Fig. 2. The stem of the funnel is slipped into a rubber tube which fits over the axle of a pulley turned by a motor. To secure even distribution of the blood about the inner wall, the separatory funnel is so arranged that its lower side is horizontal. The proper position may be attained either by suspending the neck of the funnel in a wire loop, or by laying the funnel in a horizontal trough.

[1] Fig. 1 in the accompanying paper by Van Slyke and Stadie shows an apparatus provided with a levelling scale, and a levelling bulb with side arm.

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SPIRAL BODIES IN BACTERIAL CULTURES.

BY LAURA FLORENCE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey.)

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During the winter of 1918–1919, in the course of some work with spore-bearing bacteria, spiral bodies resembling spirochetes were frequently found in cultures. When these were shown to Dr. Theobald Smith, he suggested that they were clusters of detached flagella such as had been seen by him at different times in cultures of anaerobes. Two interesting coincidences occurred at this time. The first was the receipt from an Institution of photographs of similar spiral bodies with the suggestion that they might be *Vibrio fetus* of infectious abortion in cattle, and the second was the publication of an investigation into spiral bodies in bacterial cultures by Koga and Otsubo (1919a). Since these authors have discussed the phenomenon as one hitherto undescribed and since their publication has more recently (1919b) appeared in Japan, it seems appropriate to call attention to earlier references and describe briefly the conditions under which these spiral bodies have now been found.

Loeffler (1889) first saw these spiral bodies when staining the flagella of the typhoid bacillus and the potato bacillus, but did not recognize their true nature until a year later (1890), when he found them in three different blood serum cultures of the bacillus of black leg. The latter were much larger than those seen in the typhoid cultures and he published, along with the description, a photograph of the preparation. At the same time he referred to a microphotograph, published by Fränkel and Pfeiffer, of the bacillus of malignant oedema in which spiral threads passed out from the organism just as he had found them in the typhoid bacillus. Three years later

Sakharoff (1893) described and photographed spiral bodies found in cultures of an anaerobe, *Bacillus asiaticus*, isolated by him from stools of cholera patients and grown in gelatin stab cultures. He agreed with the hypothesis of Loeffler that these were made up of clusters of detached flagella, since they could not be gotten rid of in long series of transfers and varied considerably in length and thickness. Also they could not be evolution forms of the bacteria, as dead spirals were numerous in twenty-four hour cultures. In the same year Moore (1893) wrote:

> "In the microscopical examination of well-executed preparations for exhibiting the flagella three conditions have been universally observed: (1) . . . . ; (2) there were a considerable number of detached or free flagella lying between the bacteria; and (3) the numbers of flagella on the different bacilli were not constant."

A more detailed account of these spiral bodies, also illustrated by photographs, was published by Novy (1894), when he described a new anaerobic bacillus of malignant oedema. He first found them unstained in smears stained with Gentian violet of the peritoneal fluid of guinea pigs and rabbits dead from inoculation, and then well stained in smears prepared after Loeffler's method. He found identical spirals in pure cultures of the organism and thought the nature of the media to be in some way connected with their formation. They occurred rarely in bouillon cultures. They were more numerous in gelatin cultures, most plentiful in agar cultures, and in the two last cases they were found in the condensation water. Loeffler's work, but not that of Sakharoff, was known to Novy and he confirmed the presence of these spirals in cultures of the bacillus of black leg and found them also in cultures of the bacillus of malignant oedema and of tetanus. He was not, however, like Loeffler convinced that they were clusters of flagella and he suggested the possibility of their being single deformed flagella, analogous to the involution forms found among bacteria, and named them "Riesengeisseln."

When studying the morphology of the tetanus bacillus Kanthack and Connell (1897) found two types of flagella which they named primary and secondary. Photographs of the latter show them to be similar to the spiral bodies under discussion, but the authors found them always attached to the organisms.

Malvoz (1902) working with Wathelet found spiral bodies in a culture of *Bacterium coli* isolated from the stools of a typhoid patient. Preparations stained after Loeffler's method were shown by them to Nuel (1893) who considered them identical with spirals which he had found almost ten years earlier in cultures made from a bacterial disease of the cornea. Like Novy he regarded them as individual flagella calling them "cils géants." Malvoz, however, inclined to Loeffler's view and referred to Migula's description of their formation as the best. He called them "cils composées," following the terminology of Sakharoff in preference to that of Nuel.

That these spirals were known to bacteriologists at the end of the last century is proved not only by Flügge's (1896) brief reference to them and Migula's (1897) account of their formation, but by the remark of Zettnow (1899), "Geisselzöpfe habe ich in den jungen anaëroben Culturen nicht beobachtet." In the atlas accompanying the first edition of Kolle and Wassermann (1902) were published Zettnow's photographs of small tufts of flagella from a pure culture of an unknown bacterium and of a very large tuft of flagella from *Sarcina agilis*. More recent references may be found in the texts of von Hibler (1908), of Kolle and Wassermann (1912), and of Friedberger and Pfeiffer (1919).

Koga and Otsubo (1919) while attempting to get pure cultures of smegma spirochetes, found spirochete-like spiral bodies in cultures of saprophytic bacilli. The occurrence of such forms in bacterial cultures was evidently unknown to them and the result of their investigation led them to conclude that they were "nothing more than an unusual development of the flagella or parts of the bacterial bodies." They worked chiefly with *Bacillus subtilis* but gave a list of other organisms, in cultures of which spiral bodies were also found.

For a number of years it has been the custom in this laboratory to keep certain cultures in tubes closed with sealing wax. In order to find out the general effect on culture growth of such a method a series of experiments with a number of spore-bearers was begun during the winter of 1918–1919. In the cultures of motile forms, viz., *Bacillus cereus*, *Bacillus mesentericus-fuscus*, *Bacillus mesentericus-vulgatus* (2 strains), and a bacillus isolated from the lung

of a calf and designated "Organism A," non-motile spiral bodies resembling spirochetes were frequently seen. None such were found in the cultures of non-motile forms being studied. All the cultures were grown on plain agar slants and the tubes were closed immediately after inoculation with paraffin-dipped cotton stoppers cut off level with the top of the tube and then pushed down approximately $\frac{1}{16}$ inch below the top. The mouth of the tube was flamed until thoroughly hot, when a small amount of sealing wax was placed over the stopper. This was absorbed by stopper and, when the tube had cooled, the space above the stopper was filled with sealing wax, care being taken to leave no air bubbles. Several series of cultures were also grown in bouillon sealed in the same way as the agar.

Spiral bodies were found in cultures of all five organisms, but they appeared with greatest regularity in the two strains of *Bacillus mesentericus-vulgatus*, in cultures of which Loeffler (1889) had also found them. At first it was thought that they were to be found only in sealed tubes, but later it was discovered that they were always present in the condensation water of twenty to twenty-four hour unsealed cultures of *Bacillus mesentericus-vulgatus*. They were also found in unsealed cultures of *Bacillus mesentericus-fuscus* and *Bacillus cereus* after the third day. They were seen first in hanging drops made from the condensation water, but were not found in smears made from the same and stained with methylene blue or carbol fuchsin. However, in smears stained after Loeffler's method they were always found well stained; but, if Johnston and Mack's modified method was followed they were not found, doubtless because they had disintegrated during the prolonged incubation in sterile water. Even in young cultures spirals of different sizes were seen, but very large ones, similar to those of the bacillus of black leg photographed by Loeffler, were found only in sealed cultures after an incubation period of fourteen to twenty days. In these large spirals striations parallel to their longitudinal axis were frequently seen. Their non-motility and reaction towards stains differentiated them definitely from true spirochetes and their presence, in cultures of motile organisms only, suggested a relationship with the flagella. Further, their regular absence from preparations stained after Johnston and Mack would seem to prove that they were lifeless.

DISCUSSION.

Since this phenomenon has been most frequently seen during the investigation of anaerobes, it has been thought that anaerobiosis and the formation of spiral bodies were in some way connected. It is now evident, however, that they are formed in aerobic cultures. We have found them exclusively in the condensation water of such cultures and in its rapid drying out may rest the explanation of their having been so frequently missed. Sakharoff (1893) when studying an aerobe, found them in hanging drops of the liquefied gelatin and there as in *Bacillus mesentericus-vulgatus* a stout pellicle had grown over the surface of the liquid. It may be said that under such a pellicle anaerobic conditions exist, but spiral bodies were found in the condensation water of *Bacillus cereus* and *Bacillus mesentericus-fuscus* after the third day, when no pellicles had formed. Also, both Loeffler (1890) and Moore (1893) found them in stained preparations of the typhoid bacillus made from cultures which they do not say were grown anaerobically. Koga and Otsubo (1919) found them in cultures of a number of bacilli but all were cultivated anaerobically. They further claim to have found flagella on *Pfeifferella mallei* and spiral bodies in their anaerobic cultures of this form.

In the earliest references no emphasis was laid on the nature of the media on which the organisms were cultivated. Novy (1894) was the first to suggest a relationship between the media and the formation of spiral bodies. Our findings agree with his in that spiral bodies were most abundant in the condensation water of cultures grown on agar slants and were very rare or entirely absent in bouillon cultures. We have not used gelatin. Koga and Otsubo (1919) state that spiral bodies did not develop at all in media lacking fresh protein and were never found, when the organisms were cultivated on agar. In explanation of these opposite findings it may be suggested that the Japanese workers studied only the colonies on the agar slants, in which we have never found spiral bodies, and did not examine the contents of the condensation water, where we have repeatedly found them. These authors did not specify in what part of the cultures grown on media containing fresh protein the spiral bodies were found, but Loeffler (1890) described those found in the cultures

of the black leg bacillus as lying on the surface layer of the blood serum.

To Migula's (1897) description of their formation as a mechanical process resulting from the collision of motile bacteria and the intertwining of their flagella in a circumscribed space nothing definite can be added. It is possible, however, that the nature of the media on which an organism is grown may exert a secondary influence on their formation and may explain the variation in their time of appearance in cultures of different organisms. It was thought that the viscosity of the condensation water might influence the formation of spirals, but this proved not to be the case.

SUMMARY.

Spiral bodies resembling spirochetes were found in cultures of bacterial organisms grown aerobically, as well as in those grown in a limited amount of oxygen. They were regularly present in the condensation water of such cultures.

As has been pointed out by other workers, such spiral bodies are to be distinguished from spirochetes (1) by their lack of motility, (2) by their reaction towards stains, and (3) by the impossibility of obtaining them in pure culture.

Their relationship with flagella is further proved by their presence in cultures of motile organisms only.

Their disintegration when incubated for two to three days in sterile water is evidence of their lifelessness.

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DATA ON THE DEVELOPMENT OF HETERAKIS PAPILLOSA IN THE FOWL.

BY H. W. GRAYBILL, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATE 18.

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INTRODUCTION.

The demonstration that the round worm *Heterakis papillosa* inhabiting the ceca of chickens, turkeys, and certain other birds is a factor in the production of the disease popularly known as blackhead has emphasized the importance of further knowledge of the biology of this worm. Birds other than chickens and turkeys in which it has been reported are the peafowl, guinea fowl, various pheasants, partridge, grouse, quail, bustard, and the domestic duck and goose. Experimental work in the production of blackhead by feeding ova of this parasite had already indicated that its development was in all probability direct. The work presented in this paper was undertaken to determine the course of its life cycle within the host. Artificial incubation and brooding make it possible to rear young birds away from the environment of adults. Thus the problem of control is much simplified and resolves itself largely into one of determining how long ova will remain viable in soil. The danger of wild hosts introducing the parasite will vary with localities. In the locality in which our work has been done this has not proved to be a serious factor, but nevertheless it is one that must be taken into consideration.

Railliet and Lucet[1] and Ackert[2] succeeded in infesting chickens by feeding them developed eggs. The latter author also succeeded in infesting chickens by feeding dung earthworms (*Helodrilus gieseleri*

[1] Railliet, A., and Lucet, A., *Bull. Soc. zool. France*, 1892, xvii, 117.

[2] Ackert, J. E., *Science*, 1917, xlvi, 394.

hempeli Smith) taken from a poultry yard. He interpreted the result as indicating that ova had adhered to the worms or were present in the alimentary tract, but did not believe the evidence excluded the possibility that the earthworm might act in some way as an intermediate host.

Development Outside the Host.

The fully formed ova are deposited by the female worm in an unsegmented condition in the ceca. They are elliptical in shape and measure 74 to 78 microns long and 41 microns broad. The shell is thick, resistant, and provides a market protection against external conditions. It is fairly uniform in thickness and in a number of eggs ranged from 3.3. to 3.7 microns. It consists of two layers, the inner one frequently of a pale blue color and having a thickness of half or less of that of the outer layer. Between the two layers at one pole is a minute, clear, lenticular body, the significance of which has not been determined. The ova pass out with the cecal contents and undergo development on the ground. The embryo when fully grown lies coiled within the shell and as a rule shows very little tendency to move. The ends of the body are clear and the middle region is marked by a broad column of minute, highly refractive granules. Except in rare instances, to be considered accidental, the developed ova do not hatch until they reach the alimentary tract of a host.

Experiments have been undertaken to determine the incubation period of the eggs, the longevity of the contained embryos, and the resistance of developed and undeveloped eggs to low temperatures and desiccation. These have been conducted in some instances under conditions quite different from those found on the soil, but they serve to give an approximate guide to what takes place in nature.

In observations on the development and resistance of ova both solid and fluid media were used. In the former, ovigerous females were cut up and spread over a layer of salt solution agar consisting of 0.5 per cent salt and 2 per cent agar in Petri dishes. In the latter, worms were cut up and placed in a shallow layer of physiological salt solution. Evaporation was compensated for by adding distilled water. The agar simply provided a moist substratum, with moisture and oxygen conditions somewhat different from those in salt solution cultures.

Incubation.—An agar and a salt solution culture were placed in a refrigerator, the temperature of which taken on 7 days ranged from 2.5 –8°C. For a period of 24 days the ova failed to undergo development but developed when removed to room temperature. At the same time two similar cultures were placed in a cool room. The temperature was rather uniform and considerably below that of the laboratory rooms. Taken on 8 days it fluctuated between 11.5 and 13.5°C. Development proceeded slowly and it required 38 days for embryos to become completely formed. Eggs on agar and in salt solution developed at the same rate. These observations indicate that the minimum temperature at which ova develop lies somewhere between 8° and 11.5 –13.5°C. The slow rate of development at the latter temperatures indicates that they are not far removed from the critical point.

Sets of agar and salt solution cultures were held at room temperature. There was no difference in the rate of development in the two types of cultures. Cultures held at a temperature which taken on 6 days ranged from 19–23.2° C. showed fully developed eggs in 8 days and by the 11th day all had developed. Cultures kept in a somewhat warmer room, in which the temperature taken on 6 days ranged from 22–25.6°C., were developed on the 8th day. In a salt solution culture, in which the temperature observed on 2 days was 18° and 26°C., development was complete in 9 days. Another culture, in which the temperature noted on four occasions ranged from 20–29°C., developed in 7 to 12 days. The development of the ova at room temperature ranging approximately from 18–29°C. therefore required 7 to 12 days.

Resistance of Ova to Low Temperature.—To determine the resistance of undeveloped ova to freezing, a salt solution culture was held for nearly 4 days in a refrigerator at a temperature of freezing. When removed to a warm room these eggs developed and produced normal embryos.

An agar and a salt solution culture containing embryonated eggs were placed outdoors in an unheated building for 7 days, from February 27 to March 5, 1920. The maximum and minimum temperature for the period ranged from 5–62°F. The eggs after being removed to a warm room for examination appeared normal and embryos were observed that showed movement.

Desiccation.—To study the influence of desiccation on developed and undeveloped eggs, ova were placed on the bottom of an empty

Petri dish and exposed to the atmosphere of a room. From time to time some were removed and placed in salt solution to determine their viability.

Undeveloped ova dried for a period of 16 days during October, 1920, were viable but at the next observation at the end of 41 days were no longer capable of development. At about the same time developed eggs were subjected to desiccation in a similar manner. At the end of 18 days many embryos were alive although a certain percentage had perished. At the next observation on the 49th day all were dead. In another test during October embryos were viable on the 8th day, but on the 10th day appeared to be dead.

Longevity of Embryos.—The embryo within the egg has a remarkable longevity. The thickness of the egg shell offers a protection against evaporation and unfavorable conditions.

In two agar cultures made January 23, 1920, and kept at room temperature embryos were observed to be alive as late as July 2 and September 20, respectively. After these dates no observations were made.

In two salt solution cultures, made at the same time as the above and held under identical conditions, on January 25, 1921, after a period of 12 months, although some embryos had perished, most of them appeared normal and movement was observed in certain instances.

A determination of longevity was made under conditions more nearly approaching those in nature. Salt solution cultures were made September 26 and kept at room temperature. At the end of 12 days (October 8), when the embryos were fully formed, the contents of each were transferred to soil in a small flower pot and mixed up with the surface layer. These pots were embedded part way in a pan containing soil and placed outdoors in a cage of wire netting. At intervals the pots were taken to the laboratory, the ova removed and examined with regard to the state of the embryos. It was found that they were still alive after 8 months.[3]

[3] In a field experiment reported elsewhere (Graybill, H. W., *J. Exp. Med.*, 1921, xxxiii, 667), in which young turkeys were exposed to soil that had been unoccupied for a period of 5 months beginning in January, it was found that viable ova were still present.

Course of Larvæ Within the Host.

Young chickens were fed the developed ova of *Heterakis* and killed at intervals to determine the location of the larvæ and observe their state of development. The breeds used were White Leghorn, Rhode Island Red, and Plymouth Rock. When introduced into the experiment they were 32 days old. Some controls from the same stock were provided. The cultures were made in Petri dishes by cutting up female worms, collected from chickens, in a shallow layer of physiological salt solution. They were 14 and 27 days old when fed.

The chickens had been reared in incubators and brooders and during the course of the experiments were kept indoors and protected from infestation with *Heterakis*.

The results of the examination of the chickens are given in Table I. The presence or absence of worms in the various situations is indicated by means of positive and negative signs. Unless otherwise stated the method of searching for larvæ consisted in mounting one or more samples of mucus, or contents of regions of the alimentary tract, or scrapings from an organ, and examining the same under the microscope, using a mechanical stage to cover the whole mount. The method used in examining completely any region of the alimentary tract consisted in slitting it open in a mason jar containing water. The jar was then closed and shaken thoroughly to dislodge contents from mucosa and bring them into suspension, after which the organ was removed and washing, sedimenting, and decanting were continued until the supernatant fluid was clear. Then the sediment was placed in Petri dishes and examined under a low power of the microscope. In examining an entire organ such as the liver, it was ground up in a mortar, then suspended in water, and poured through a tea strainer to remove the larger particles. The filtrate was then washed, sedimented, and examined according to the method already described.

The chickens were killed by chloroforming and the examinations made as rapidly as possible. They were killed after periods varying from $2\frac{1}{2}$ hours to 57 days.

In an autopsy made on a chicken $2\frac{1}{2}$ hours after receiving ova the eggs were still in the crop and none had hatched. After 24 hours apparently all had hatched. Larvæ were found only in limited num-

TABLE I.

No. of chicken.	Period after feeding ova.	Esophagus.	Crop.	Proventriculus.	Gizzard.	Small intestine.	Large intestine.	Ceca.	Cecal wall.	Lungs.	Liver.	Spleen.	Body cavity.	Remarks.
	hrs.													
320	2½	—	Unhatched ova.	—		—*								Chicken was given no food after receiving eggs.
	days													
321	1	—	—	—	—*	—*	Many.	Many.			—†	—†	—*	Organs normal.
275	1	—		—		Several.		+	—‡	—	—‡			Feces passed on autopsy table washed and sedimented. No larvæ or ova found.
322	2			—		Two.*	—	Many.*	—§					All organs normal.
276	2					—	—	+	—‡		—‡			" " "
277	3							+	—‡		—‡			Organs normal except one lung congested.
323	4	—	—	—		—*	—*	+	—§	—	—†		—*	All organs normal.
324	7	—	—	—		—*	—*	Many.	—§		—†		—*	Walls of ceca possibly slightly thickened and contained white specks. Mucosa congested. Other organs normal.
278	7							+	—‡		—‡			All organs normal except one lung congested.
325	9	—	—	—		—*	—	+	—§	+	—†			Mucosa of ceca congested. Scraping from congested place in lung contained anterior half of larva. Other organs normal.
279	10							+	+‡		—‡			Blackhead. All organs except ceca were normal.
280	11							Many.	+‡		—‡			Blackhead. All organs normal except ceca.

326	11	–	–	–		–*	–	+		–	–†		All organs normal except ceca.
281	14							+	+‡		–‡		Blackhead. All organs normal except one cecum. Mucosa covered with hemorrhages.
282	14							+	–‡				Blackhead. All organs normal except ceca.
327	16	–	–	–		–*	–	Many.	–§				Ceca showed certain abnormalities but other organs were normal.
285	18							+	–‡		–‡		All organs normal.
328	21	–	–			–	–	–*	–§				" " "
286	23							+	–‡		–‡		" " "
287	28							+	–‡		–‡		Mucosa of one cecum not normal.
288	29							+*	–‡		–‡		Mucous folds of ceca absent in places. Other organs normal.
332	35							7*					All organs normal.
335	49							–					" " "
336	51							1*					" " "
338	57							8*	–§				" " "

Three controls were killed, two on the 17th day and one on the 30th day. The organs were normal. The contents of the ceca were washed and sedimented but no larvæ were found.

* Contents washed, sedimented, and examined.

† Organs ground up, washed, strained, and sedimented.

‡ Fixed and stained sections.

§ Piece of fresh wall examined microscopically under pressure between two slides.

bers in the posterior part of the small intestine but in large numbers in the large intestine and ceca. After 2 days in one instance two larvæ were found in the small intestine but none elsewhere except in the ceca. The fact that larvæ in one instance were found in great numbers in the large intestine a day after feeding suggests that this is a site where they collect to migrate to the ceca. The point in the alimentary canal where the ova hatch has not been determined. It apparently does not take place in the crop. It seems probable that the small intestine is the site where the embryos emerge. No larvæ were found in lungs, liver, spleen, cecal wall, or body cavity. The larvæ in general had the same character as embryos within the egg.

In summarizing the observations on chickens killed the 3rd day and thereafter, it should be stated that worms were found only in the ceca and in three instances in the walls of the same. In one chicken (No. 325) a fragment of a larva was found in a scraping from a congested lung, but as no others were observed in further mounts, it seems most probable that this had been introduced by accident, possibly on instruments. Examinations in various instances of regions of the alimentary tract other than the ceca, of the lungs, the liver after being ground up, washed, strained, and sedimented, and of washings from the body cavity were uniformly negative for larvæ. Microscopic examination of the washed cecal wall were likewise negative. In two out of the twenty chickens examined after the 3rd day it was not possible to find worms in the ceca. Three controls examined were negative.

A surprising result of the feeding of ova was the light infestation resulting. It is believed that each bird received at least several hundred eggs, yet the number of worms found in certain instances was small. While the worms are still microscopic or visible to the naked eye with difficulty, it is not easy to form an estimate of the numbers present. However, in the case of Nos. 321, 322, 323, 324, 280, and 327, all killed on or before the 16th day, many larvæ were present. The presence of only three larvæ in a cecum of No. 286; two larvæ in the case of No. 287; and seven and eight worms in both ceca of Nos. 332 and 338 respectively, is evidence of the small infestation resulting. There are in all probability a number of factors involved in this result. Among these may be mentioned the artificial method of collecting

and incubating the ova. It is conceivable that this might have a deleterious influence on the vitality of the larvæ. Also the abnormal condition of the ceca and their contents in cases that develop blackhead might have an unfavorable influence on the larvæ.

It will be noted that worms reached maturity in 57 days. Taking 7 days, which was observed in experiments at room temperature as the shortest time for embryos to become fully developed, as the minimum period for development outside the body, the entire cycle would require 64 days.

Development of Larvæ (Figs. 1 to 11).

Embryos may be freed from ova by pressing on the cover-glass of a fresh mount and crushing the shell (Figs. 1 and 4). If uninjured on emerging they show an active wriggling motion. The maximum width occurs towards the anterior end and the body tapers in both directions, anteriorly to a rather blunt, rounded point, and posteriorly to a long, fine point. The length ranges from 290 to 340 microns, and the maximum width from 14 to 15.6 microns. The anus is situated 45 microns from the posterior end. The anterior end is nude and the mouth is subterminal, being located a little dorsal to the tip of the body. Lateral wings are present, and viewed from the side of the worm they appear as lines, most prominent in the middle region of the body and gradually shading out toward the extremities. The intestine is marked by a band of highly refractive granules extending from a point anterior to the middle of the body to near the anus. The esophagus is slightly bulbous, the bulb being elongated. The cuticula at the anterior tip of the body is slightly thickened.

Larvæ 1 and 2 days old show little change from the embryo just emerged (Figs. 2 and 3). The mouth is still subterminal. The rectum was wedge-shaped and in some instances it alternately expanded and contracted like a pulsating vacuole. A 2 day larva measured 320 microns long and 17 microns broad, and one at 3 days was 338 microns long.

Larvæ at the end of 7 days showed a marked growth (Fig. 7 and same stage as shown in Fig. 8). One measured 0.5 mm. in length and 28 microns broad. Those studied on the 9th and 10th days were somewhat more advanced (Figs. 8 and 9.) One measured 3 mm. In

some instances a pair of small round papillæ were observed for the first time on the anterior end opposite each other.

Larvæ collected on the 14th day were round and smooth on the anterior end with the exception of the presence of the papillæ already mentioned. Five ranged in size from 2.9 to 3.8 mm. A number of rather young larval stages were observed, a circumstance noted also on other occasions, indicating a considerable variation in the rate of growth.

By the 16th day lips were seen forming within at the anterior end, and chitinous structures similar to those of the adult were present in in the esophagus or in process of formation The papillæ already noted on the anterior end were present. These are always seen in optical section of the worm on either side some distance from the mouth, but there may be others not visible on the upper and lower surfaces. At about this time when the lips become free a molt takes place, but specimens showing this have not been encountered. Larvæ measured ranged from 3.2 to 3.9 mm. long.

On the 18th day specimens were found showing further development. Sexes were differentiated, the males being provided with a bursa and papillæ. Larvæ ranged in size from 2.6 to 4 mm. long. On the 29th day worms had reached a maximum of 4.5 mm. in length. By the 35th day some worms were about half grown, and by the 57th day they had apparently reached full development.

SUMMARY.

In observations on the development of the ova of *Heterakis papillosa* in cultures, it was found that they failed to develop at a temperature ranging from 2.5–8°C., but developed slowly at a temperature of 11.5–13.5°C. The minimum temperature for development seems to lie between 8° and 11.5–13.5°C. At temperatures ranging in various cultures from 18–29°C. ova developed to their final stage in 7 to 12 days.

Undeveloped ova subjected to a freezing temperature for a period of 4 days were viable at the end of that time. Fully developed ones remained alive when exposed out of doors for a period of 7 days at a temperature ranging from 5–62°F.

Undeveloped ova survived desiccation at room temperature for a period of 16 days, but not for 41 days. Fully developed eggs were alive after desiccation for 18 days, but not after 49 days. In another instance they were no longer viable after 10 days.

Embryos within ova kept in physiological salt solution at room temperature survived during a period of a little over 12 months. Fully developed ova kept in soil outdoors under circumstances approaching natural conditions contained living embryos after a period of 8 months.

From a study of a series of artificially infested chickens killed at short intervals it appears that the ova of *Heterakis* hatch in the small intestine and the larvæ pass by way of the small and large intestines to the ceca where they undergo development to maturity. Larvæ found in the mucosa of the ceca were not in an encysted condition.

Feeding of numerous artificially incubated ova may lead to a light infestation, the cause of which has not been definitely determined.

A period of 57 days was required for larvæ to reach maturity in a host. The entire cycle from egg to adult requires a minimum time of about 64 days.

A brief study of the growth and development of larvæ within the host has been made. No evidence was found of a migration through the tissues. A few penetrate into the mucosa of the ceca.

EXPLANATION OF PLATE 18.

Drawings made with camera lucida from fresh material.

FIG. 1. Embryo from an egg in a culture. Magnification about 196.

FIG. 2. Larva, 1 day old. Rendered immobile by adding alcohol and glycerol to mount. Magnification about 196.

FIG. 3. Larva, 2 days old, showing lateral wing. Magnification about 196.

FIG. 4. Anterior end of specimen shown in Fig. 1. Shows esophagus and subterminal position of mouth. Magnification about 834.

FIG. 5. Anterior end of a larva 3 days old. Magnification about 834.

FIG. 6. Anterior portion of body of a larva 4 days old, showing bulbous esophagus and anterior end of intestine. Magnification about 387.

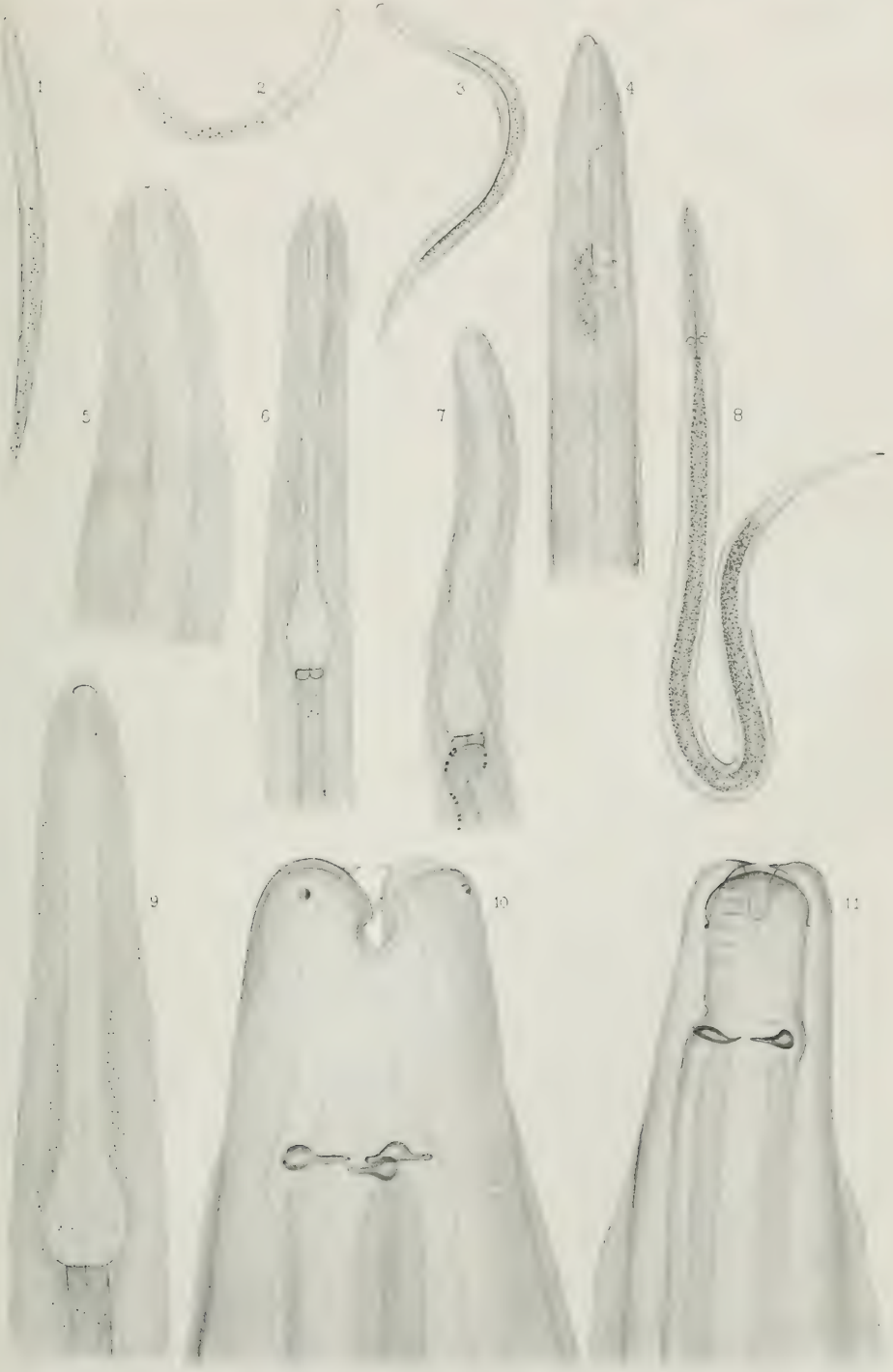
FIG. 7. Anterior region of body of a larva 7 days old. Shows bulbous esophagus. Magnification about 425.

FIG. 8. Larva 10 days old. Shows bulbous esophagus and intestine crowded with granules. Magnification about 46.

FIG. 9. Anterior end of same larva. Shows a differentiation of pharynx. Magnification about 182.

FIG. 10. Anterior end of worm 29 days old. Shows the two ventral lips with teeth and papillæ, the pharynx and chitinous structures of same, and of esophagus. Magnification about 660.

FIG. 11. Anterior end of a worm 35 days old. Dorsal view showing three lips. Magnification about 387.



(Graybill: *Heterakis papillosa* in the fowl.)

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ON THE CYTOLOGY AND LIFE HISTORY OF THE AMOEBAE.

With Descriptions of Two New Species.

By R. W. GLASER.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Plates 1 and 2.

INTRODUCTION.

It was shown by Graybill and Smith (1920) and Smith and Graybill (1920) that blackhead in turkeys and chickens could be experimentally produced by feeding the embryonated eggs of the nematode, *Heterakis papillosa*. They had frequently encountered protozoa in the cultures in which the ova were developing, and it was considered advisable to determine whether or not these organisms represented some stage of the protozoon responsible for the disease. These results led the writer to make a careful study of the protozoan fauna encountered in Heterakis, as well as in the embryonated egg cultures. These investigations disclosed nothing of importance concerning blackhead, but the amoebae studied revealed a number of new and interesting cytological and life history characters.

The best method for obtaining the amoebae under discussion consists in removing adult Heterakis from the ceca of a variety of healthy chickens. The worms are thoroughly washed in sterile physiological salt solution, and sedimented several times. They are then cut up and the material incubated in physiological salt solution for fifteen to thirty days at room temperature. During the summer of 1920, nineteen cultures were thus prepared from different lots of chickens.

On examining incubated Heterakis cultures in from two to three weeks, it was found that besides worm fragments, embryonated Heterakis ova, and bacteria, the cultures nearly always contained the trophozoites and cysts of amoebae. Two species were found in the salt solution cultures either together in the same culture or separately in different cultures. The amoebae were easily recognized, particu-

larly the cysts resting on the bottom of the Petri dish, but when the writer was in doubt he placed a worm fragment under the microscope. Such a fragment usually proved to contain many amoebae which probably fed upon the countless bacteria found within the dead worm tissues. The two forms were the only amoebae which developed within the salt solution cultures. By the use of the methods of Frosch (1897) and Walker (1908), the amoebae were isolated and grown on Musgrave and Glegg's medium, 0.7 of normal alkaline, in association with cultures of *Staphylococcus pyogenes albus*.

For the study of some of the stages the writer found Walker's hanging plate method indispensable, but found, and Walker also noted, that the amoebae will usually not pass through their complete life cycle on the hanging plate but take the short cut from cyst to cyst merely through the trophozoite stage. In order to obtain all of the stages, one must also resort to the Petri plate cultures.

The various stages that were fixed and stained were always compared with the living material of similar stages that grew on the hanging plate cultures. One can obtain a much more accurate picture of the organisms from the hanging plate cultures than by removing some of the organisms from Petri plate cultures to some liquid medium like Ringer's, Locke's, or physiological sodium chloride solution. Should the latter procedure be necessary, one must be careful to use a solution strictly isotonic to the liquid constituents of the protoplasm, otherwise one produces certain deformations of the organisms, manifested by plasmolysis or extensive grosser granulations and other cytologic structural changes that do not normally exist.

When certain stages fail to develop on the hanging plate, it is necessary to consult the Petri plate cultures and examine the organisms in some carefully balanced non-toxic solution, but the best results are obtained by removing the stages from the Petri culture and by immersing them immediately in some fixative. Fixation was accomplished either by means of Zenker's or Schaudinn's solutions. Mallory's chloride of iron hematoxylin was used extensively after Zenker's fixation, but some stages of the amoebae were better elucidated by means of Delafield's or Heidenhain's iron hematoxylin after Schaudinn's corrosive sublimate alcohol mixture.

Vital staining (methylene blue and especially neutral red (1.3,000 to 1:10,000) was used during the studies on cysts and the method of sporulation. This method cleared up much that remained obscure when fixing and other staining methods were used.

It seems advisable to tentatively place the two organisms studied by the writer in the genus Amoeba rather than in the genus Entamoeba for reasons which will become apparent later. However, it might be well to state that no evidence was found to support the view that the organisms to be described multiply or pass through any stages within the intestines or ceca of chickens. Recently Tyzzer (1920) described two true host inhabiting in chickens and turkeys. He did not report stages of the organisms described in this article, so it seems probable that the amoebae here described are what are known as intestinal passers, possibly derived from the soil, and remain in the encysted stage until eliminated with the excrement.

Amoeba commutabilis sp. nov.

This amoeba when cultivated on the hanging plates (sealed depression slide cultures) usually presents only the simple amoeboid and cyst stages. If the medium on the cover slip is inoculated with cysts and some food bacteria (*Staph. pyogenes albus*), the trophozoites appear in 48 hours at room temperature or sooner at 32°C. The trophozoites are exceedingly sluggish. The cytoplasm is clear, faint and finely granular. There is no distinct differentiation between endo- and ectoplasm except when the pseudopodia form. The formation of the pseudopodia is eruptive, and when they erupt they often run along the periphery of the amoeba in a wave-like motion. There seems to be no pronounced flowing of the endoplasm. Sometimes the pseudopodial processes are quite pointed, but usually blunt. They are always rather short. A few food vacuoles are present containing bacteria during the feeding phase. A small contractile vacuole is present. The nucleus is clear and faint with the chromatin concentrated within the karyosome during the feeding and growing phase. Plate I, Figs. 1, 2, 4 and 5 represent living amoebae of the growing and feeding phase. Figures 1 and 2 represent amoebae examined in isotonic solution and showing no clear differentiation between endo- and ectoplasm. The ectoplasm in these cases appears granular.

Figures 4 and 5 represent amoebae examined on depression slide cultures showing differentiation between endoplasm and pseudopodial ectoplasm. When conditions become unfavorable on the depression slides, the amoebae first become quiescent, then free themselves of waste products, assume a round form and encyst (Fig. 6). Encystment on the depression slides occurs in four or five days. The early cysts still show the chromatin concentration represented by the karyosome which is surrounded by a clear, distinct, but chromatin free nucleus. The cysts show only one wall when examined on the depression slides or in a strictly isotonic solution in which no plasmolysis occurs. The resting trophozoites measure 5 to 8μ. The cysts measure 6μ.

The agamic multiplication of the trophozoites can best be followed by roughly detecting the nuclear and karyosomic changes on the depression slide, and by immediately removing the cover slip containing the agar film culture, immersing in the fixative, and staining carefully by one of the histologic methods outlined previously.

Neither a sexual phenomenon nor the suggestion of one was encountered. The trophozoites multiply by a sort of primitive mitosis known as promitosis, or the division of the karyosome. Hartmann did much work on this method of cell division, but certain important details found in this amoeba seem to differ from methods heretofore described. In the most primitive protozoa showing this form of nuclear division, Hartmann found that the karyosome contains all the chromatin and nuclear material and is submerged in a zone of nuclear sap. No definite nuclear membrane exists. In higher forms chromatin and supportive structures are found within the nucleus proper and a definite nuclear membrane appears.

During the course of the studies on *A. commutabilis*, it was found that at certain times the amoebae could be placed within the primitive group and at times within the modified group. At a certain stage the karyosome contains all the chromatin and is situated in clear nuclear sap containing no supportive structures. At another stage the karyosome gives off chromatic material (chromidia) into the nucleus or the karyosome may disappear entirely, all the chromatic material being contained within a large diffuse nucleus. In still other stages the nucleus disappears, the chromatic material diffusing

out into the cytoplasm, and later, at another stage, to be reorganized and incorporated into spore material. The presence of a nuclear membrane is never much accentuated at any stage.

The changes observed by the writer and outlined above have been attributed to physiologic cyclic changes and, in this case at least, he is unable to decide upon the phylogenetic status of the organism based on the structure of the nucleus and karyosome.

The trophozoites preparatory to division come to rest and assume a more or less round form (prophase). The karyosome then slips into two equal halves (Pl. I, Figs. 7, 8, metaphase). A centriole was never seen. In the next stage or early anaphase (Fig. 9) the nucleus is greatly hypertrophied and contains a spindle with the karyosomic halves diverging. In a late anaphase (Fig. 10) the nucleus has disappeared; an interzonal region shows clearly between the two karyosomic halves and there is a weak suggestion of a modification of the astral ray phenomenon. Figure 11 represents the telophase with the organization of two nuclei containing each a karyosome. This condition is later followed by cell division into two daughter amoebae which separate and move off one from another. The agamic method of reproduction in this amoeba seems not to be preceded or accompanied by any phenomenon of reduction. At this time no purification of the karyosome occurs as recognized by the formation of chromidia in the nucleus. The nucleus during its existence up to late anaphase remains perfectly clear.

Another method of multiplication is resorted to in this species, namely, through the formation of spore-like structures homologous to those found by Schaudinn in *Entamoeba histolytica*, and by Walker in a number of amoebae parasitic within the intestinal tract. The details of this sporulation process in *A. commutabilis* again differ from the previously described cases. Sporulation occurs rarely on the depression slides (hanging plates), but most frequently in the Petri plate cultures. Indeed, even in such cultures very often the cyst, trophozoite, cyst cycle may be all that is revealed and the other stages are entirely omitted. The conditions under which these phenomena occur or not are obscure. Physical factors may be involved or certain processes may occur only after a definite number of agamic reproductive generations. Sporulation, when it does occur within a Petri

plate culture, is quite universal within the particular culture, and one has no difficulty in tracing certain details of the process and some of the interesting ones that follow.

As was pointed out previously, the early cyst assumes the aspect illustrated on Plate I, Fig. 6. The amoeba assumes a round form, with the chromatic material concentrated as a karyosome and submerged within a clear nucleus. The time required for certain changes varies so much that it is impossible to give any specific data on this point. Generally speaking, in about 12 hours the karyosome begins to purify itself by throwing out or secreting chromoidia into the nucleus (Fig. 12). On account of the minuteness of the structures involved in the process, it is impossible to affirm with any conviction that the chromidia are ultimately incorporated within the forming spores and that later some of them are also used during the synthesis of the new trophozoite nucleus. However, the process as it was observed is very suggestive of these theoretical assumptions.

About 6 hours after the stage described above the condition represented by figure 13 is found. The karyosome has vanished or has been entirely resolved into chromidia, which are now represented collectively by a large diffuse nucleus. Still later (Fig. 14) the nucleus disappears and minute, deeply staining chromatic bodies are found scattered through the cytoplasm. Later stages (Figs. 13 to 18) follow rapidly; some of them seem to consume only a few hours. Figure 15 shows the growth of the deeply staining bodies which are the so-called spores. If this stage is transplanted to fresh media containing living bacteria, the trophozoite emerges from the cyst in about 12 hours. From cyst to trophozoite, when a fresh cyst is transplanted, consumes in all about 48 hours. The newly emerged trophozoite still contains no nucleus, only spores varying in numbers from five to eight (Fig. 16). The spores also vary considerably in size from quite small ones to some spproaching the size of the karyosome of this species. At a later stage (Fig. 17) the trophozoite shows a nucleus and karyosome. The spores are now extruded within a few hours. At this period the writer found no large amoebae undergoing cytolysis, so it appears that the adults after spore extrusion continue to live and divide agamically.

The method of sporulation described does not harmonize with Walker's (1908) general statement that "sporulation, like other vital processes, ceases with the encystment of the amoebae." Also in no case was Walker able to observe any nuclear changes preceding or during the process of sporulation. Nuclear changes do precede and accompany sporulation in the species here studied, and in this fact we seem to agree more nearly with Schaudinn (1903), who found in the case of *Entamoeba coli* that profound nuclear changes and a sort of sexual process resulting in eight daughter nuclei and in the corresponding number of amoebae accompany the process of encystment. It seems to be unsafe to make a general statement covering all of the manifold and complicated processes occurring within a class of organisms represented by so many species and still so little understood. One set of cytological and other phenomena may be observed in one species, whereas the reverse or a totally different set of processes may become apparent in another.

In *A. commutabilis* the spores soon develop on the Petri plate cultures if conditions are favorable. Often they fail to develop at all, and it is necessary to make many cultures in order to procure a number that are favorable for study. Development seems to be direct (Fig. 18) as Walker found in other species. At first the spore resembles the karyosome of the amoeba in size and structure. Many individuals show a densely staining periphery and a lighter center. In a short time, the spore enlarges and becomes delicately and uniformly granular throughout with a small number of deeply staining granules in the cytoplasm. The next stage assumes a more amoeboid aspect larger in size with the organization of chromatic material near the center. The final stage in this series is represented by a small amoeba with a well organized nucleus and karyosome. At this time the organism shows amoeboid movement.

The possibility exists that the deeply staining bodies encountered and termed spores may not be spores at all, but deeply staining chromatic material with a small enveloping layer of cytoplasm which the writer was unable to demonstrate due to faulty technic. In comparison with certain cytologic changes occurring in *E. coli* and *E. histolytica*, it is conceivable that the deeply staining bodies really represent aggregations of chromidia which later surround themselves with a

small amount of cytoplasm. These are extruded as very minute amoebae, at first showing no amoeboid movement. The parent *A. commutabilis* does not fragment; the young amoebae or spores are extruded. No autogamous processes preceding the formation of these so-called spores was observed.

Another stage in the complicated life cycle of this amoeba was found, and this stage was the one which really attracted the writer to the interesting potentialities of this species. Budding occurs in a large number of cultures under the proper conditions. Agamic simple and multiple budding was observed by Schaudinn in *Entamoeba histolytica*. Schaudinn not only observed this phenomenon in material taken from the intestinal lumen of cases of dysentery, but also observed it between the cells of the intestinal epithelium. Schaudinn considered that amitotic division of the nucleus accompanies this method of multiplication.

In so far as it is possible to determine, Schaudinn seems to have been the only worker who observed budding in amoebae, and when this condition first appeared on the *A. commutabilis* plates a contamination with a saccharomycete or with the yeast-like stages of some of the higher fungi suggested itself. After repeated isolations and platings the writer was able to convince himself that budding occurs at times within the species studied.

Budding when it does occur in *A. commutabilis* appears about 12 to 24 hours after the so-called spores have given rise to small amoebae. The small amoebae at first grow a bit and divide a number of times (Fig. 19). Soon after one begins to recognize such forms as are represented by figure 3 (from living specimen) and figure 20 (fixed and stained). A small bud will be found beginning to constrict off from the parent cell and without the suggestion of any nucleus. The nucleus of the parent cell shows the presence of a karyosome and chromatin granules within the otherwise clear nucleus. These chromatin granules or chromidia undoubtedly diffuse out into the cytoplasm and some of them probably reach the bud before constriction occurs. Figure 21 shows another case of budding with the karyosome in a curious, stellate, cyclic phase. Figure 22 represents a larger amoeba budding and also showing pseudopodial activity. By examining a number of budding cultures in the fresh condition with the aid of the warm stage, the writer has frequently observed budding

accompanied by slight and slow pseudopodial activity in certain larger individuals. Generally speaking, however, budding is a function of the small amoebae and usually disappears entirely when they become large. Figure 23 is difficult to interpret. This case may represent unequal division of a small amoeba or rather equal budding or, lastly, it may represent an abnormal condition. Multiple budding, as observed by Schaudinn in *E. histolytica*, does not occur.

After the buds have formed they are gradually constricted off. The time required for this and some of the other processes has not been noted, for the reason that budding does not occur on the hanging plate cultures which lend themselves better to time observations. Figure 24 represents a number of stages in the development of the bud to the small amoeba. The first stage looks much like the adhering bud. It is more or less round and finely granular with no nucleus. Later an organization of chromatic material occurs and the bud begins to assume an amoeboid form. On examining the Petri cultures still later, one finds typical small amoebae (Fig. 25) showing a clear nucleus with the deeply staining karyosome.

The stages represented by figures 26 and 27 are also difficult of interpretation and I hestitate to offer one. These stages were rarely found in resting amoebae, not cysts, and probably represent nuclear cyclic changes. The ray-like structure (Fig. 26) is not the opening in a cyst through which an amoeba has emerged, but a nucleus with peripheral chromatin and sending chromatic rays into the cytoplasm.

Amoeba vegetabilis sp. nov.

This species is an exceedingly active form when examined on the hanging plate cultures. A distinct differentiation between endo- and ectoplasm exists (Plate 11, Figs. 28, 29, 30 and 31) except when in the resting condition (Figs. 35 and 36). A continual streaming of the entire protoplasm occurs. The endoplasm is granular and contains many ingested bacteria. The formation of the pseudopodia is not eruptive as in the preceding species, but they form in an even flowing manner. One or two broad pseudopodia occur which may terminate bluntly (Fig. 28) or in a pointed or saw-toothed fashion (Figs. 29 and 30). A contractile vacuole is present, and an interval of approximately 65 seconds intervenes between two pulsations. After con-

traction the new vacuole arises again at two or three minute points that increase in size and suddenly flow together into one large vacuole which soon contracts. The nucleus is of the karyosomic type with all the chromatin concentrated in the center and submerged in a clear surrounding area. This type of nucleus seems to exist during the greatest feeding and digestive phases when (Figs. 32 and 33) the chromatic material is diffuse throughout and no karyosome is present. No especial study of the agamic multiplication of this amoeba was made, but since the nucleus is of the karyosomic type a form of mitosis seems probable.

This amoeba was also studied on Petri plate cultures and the vegetative or agamic method of reproduction appeared to be the only one. The formation of spores or a process of budding was never observed. The cyst, trophozoite, cyst cycle, consuming from 5 to 9 days, constituted the entire history on the media used. When cysts are placed on fresh media in the presence of suitable bacteria (*Staphylococcus pyogenes albus*) and kept at room temperature, the trophozoites emerge in from 24 to 48 hours.

When fixed and stained properly, the cytoplasm of the trophozoites appears to be highly vacuolated (Figs. 32, 33, 34 and 35). Large food vacuoles are also in evidence containing phagocytized bacteria. Figure 33 represents the only fixed specimen found showing a large ectoplasmic pseudopodium. Figure 35 represents a round resting stage.

When the amoeba begins to encyst, it comes to rest, becomes round and the cytoplasm loses its vacuolated appearance, becoming granular (Fig. 36). No karyosome can be seen, the entire nucleus staining. After about 12 hours or more the amoeba becomes polygonal or irregular, a contraction of the protoplasm occurs and a two-walled cyst is formed (Figs. 37, 38 and 39). In fully formed cysts the amoeba proper may appear to be polygonal and rather regular (Figs. 37 and 38) or it may assume a stellate or irregular form with rounded, blunt angles (Fig. 39). At times the encysted amoeba follows closely the outline of the cyst wall proper, which is usually quite irregularly scalloped. The angles of the amoeba do not touch the cyst wall. The cyst wall proper is clear and hyaline. After the emergence of the a ' ɹ the empty cysts appear yellow in color. One nucleus

is present within the cyst. The average resting *A. vegetabilis* measures 10 to 13μ. The nucleus measures 2μ and the cysts 10μ.

The amoeba described as *A. vegetabilis* somewhat resembles Walker's *A. intestinalis*. Several diagnostic distinctions exist, but the principal ones deal with the differentiation of endo- and ectoplasm, with locomotion and reproduction. In *A. intestinalis* the ectoplasm is scarcely apparent except in the pseudopodia; in *A. vegetabilis* (Figs. 28, 29 and 30, from living specimens) the ectoplasm appears usually to envelop the entire amoeba. Furthermore, *A. intestinalis* is not very active, whereas *A. vegetabilis* is exceedingly active. *A. intestinalis* forms spheroidal spores 0.5 to 1.5μ, whereas *A. vegetabilis* does not form any spores.

EXPERIMENTAL DATA.

Since Tyzzer and Fabyan (1920) were able to produce certain lesions of blackhead in young turkeys, chickens, and pigeons by subcutaneous and intramuscular inoculation of tissue from liver lesions of acute cases, it seemed advisable to inoculate these birds with cultures of Heterakis ova, and also with the two species of amoebae isolated and described in this paper. Following the directions of the above named workers explicitly, young turkeys, 5 to 9 days old chickens, and pigeons were inoculated. Small, white, indurated masses or, in some cases, quite extensive reactions were obtained after a few days in the connective tissue under the skin. In no case, however, were any of the lesions of blackhead reproduced. Sectioned material was studied extensively, and it was found that the amoebae associated in the Heterakis cultures and used for these experiments were not modified to resemble *Histomonas meleagridis* Tyzzer (*Amoeba meleagridis* Smith) after gaining admission to the bird tissues. Usually the amoebae were killed off by the tissues or, when found, resembled the amoebae as they appear in culture.

Whereas it has been proven by Graybill and Smith (1920) that one of the factors for the production of blackhead in turkeys and chickens is present in the Heterakis cultures in the form of the embryonated worms, the results of the experiments here briefly outlined seem to show that the protozoan factor is missing in such cultures.

SUMMARY.

1. Commonly one of two species of amoebae or both species of amoebae developed within embryonated Heterakis salt solution cultures. The amoebae were cultivated in pure cultures, and described as two new species, *viz.*, *A. commutabilis* and *A. vegetabilis*.

2. In *A. commutabilis* a modification of the promitotic method of division is described. In this same amoeba interesting nuclear cyclic changes were observed.

3. New cytologic details accompanying and following sporulation were elucidated in *A. commutabilis*. A process of budding was discovered, accompanied and followed by nuclear cyclic changes. A sexual process was not observed.

4. Agamic division, a kind of sporulation, and budding constituted the methods of multiplication in the life history of *A. commutabilis*.

5. Agamic division, probably promitotic, was the only method of multiplication found in *A. vegetabilis*.

6. Turkeys, chickens, and pigeons when inoculated with Heterakis cultures containing one or both species of amoebae, or when inoculated with pure cultures of the two species of amoebae, failed to develop lesions of blackhead.

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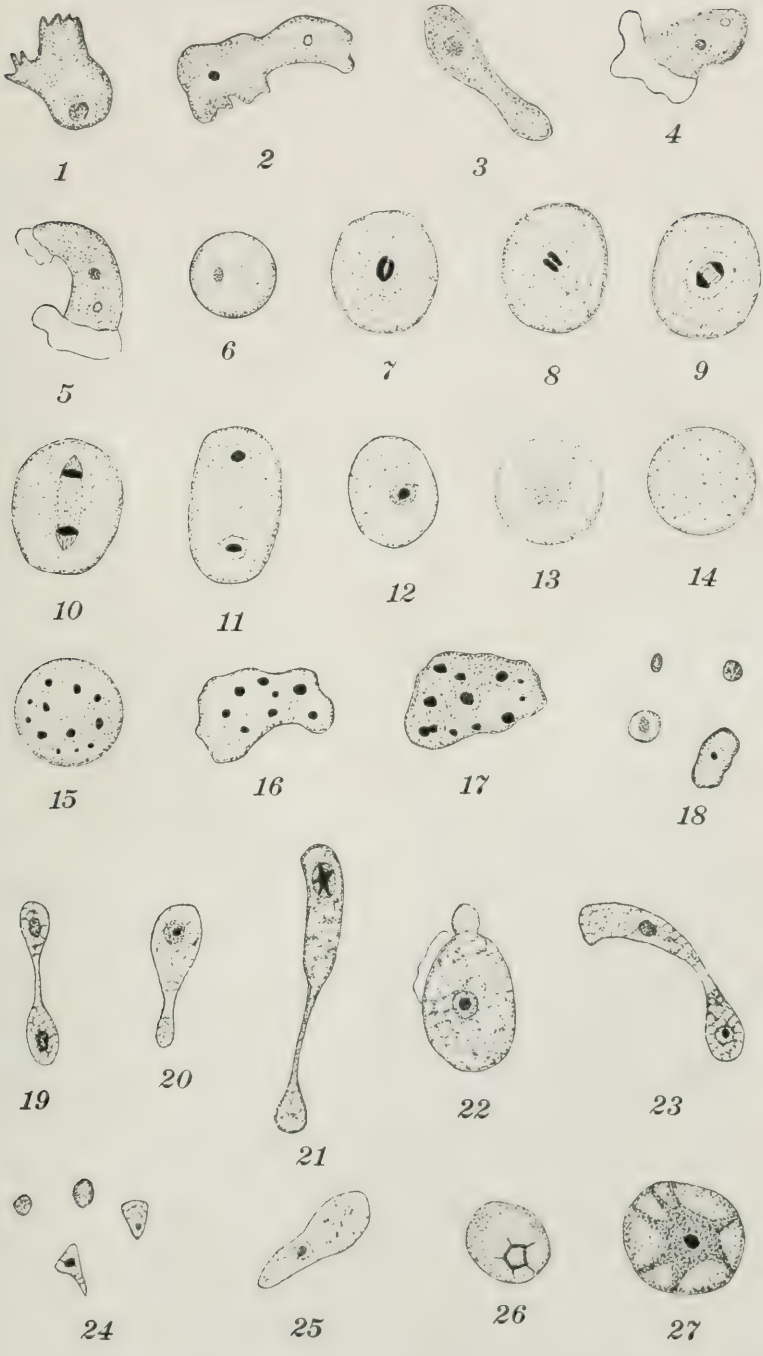
EXPLANATION OF PLATE I.

Plate I.—*Amoeba commutabilis*. × 813. Figs 1-5, Living trophozoites. 6, Cyst. 7-11, Fixed and stained preparations showing promitotic division. 12-18, Process of sporulation. 18, Development of spore. (Fig. 12, fixed and stained. Figs. 13-18, stained intravitally.) 19-24, Fixed and stained preparations showing process of budding. 24, Development of bud. 25, Young trophozoite derived from bud. 26-27, Uninterpreted nuclear cyclic changes.

EXPLANATION OF PLATE II.

Plate II.—*A. vegetabilis*. ×813. Figs. 28-31, Living trophozoites. 32-34. Fixed and stained trophozoites. 35, Resting stage. Fixed and stained. 36, Early cyst. 37-38, Types of mature cysts.



(Glaser: Cytology and life history of amoebæ.)

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(Glaser: Cytology and life history of amoebæ.)

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THE USE OF SODIUM SULFATE AS THE GLOBULIN PRECIPITANT IN THE DETERMINATION OF PROTEINS IN BLOOD.

By PAUL E. HOWE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, September 1, 1921.)

In connection with a study of blood under normal and pathological conditions we proposed to use the method of Cullen and Van Slyke (1), for the determination of fibrin, globulin, and albumin nitrogen of blood plasma. A serious objection to the method is the use of ammonium sulfate as the globulin precipitant. This is so for two reasons, (*a*) because of the use of an ammonium salt which must be removed before determining the globulin nitrogen and (*b*) because of the physical difficulties involved in the removal of this nitrogen with magnesium oxide; particularly with reference to the "bumping" of the mixture. Both of these factors were realized by Cullen and Van Slyke, who, however, showed their method to be accurate, and believed that they had found a way of preventing the extreme bumping. We have corroborated their method as far as duplication of results is concerned but found that Merck's highest purity magnesium oxide is not always suitable for these determinations. Satisfactory results were obtained only by constant shaking of the Kjeldahl digestion flask during the distillation of the ammonia and the early part of the digestion.

The use of a non-nitrogen-containing precipitant in place of the ammonium sulfate, it seemed, would remedy both of the defects indicated above. The salt most commonly replacing ammonium sulfate for the precipitation of the total globulins is magnesium sulfate. Sodium sulfate has also been shown to be a satisfactory precipitant for globulin. Preliminary trials with these two salts indicated that, with regard to clean precipitation and rapid filtration, sodium sulfate was far superior to magnesium sulfate. When magnesium sulfate is

used to precipitate proteins, the precipitates are gelatinous in character and the solutions filter slowly. The chief objection to the use of sodium sulfate is the necessity of working at temperatures above 34°C. for precipitation at the highest concentrations of the salt. The solubility of $Na_2SO_4 + 10\ H_2O$ increases gradually up to approximately 10°C. and then rapidly to 34°C.; above 34°C. the anhydrous salt is in equilibrium with water and the solubility of the salt decreases gradually. Working at incubator temperatures conditions with regard to solubility are those which obtain in general with magnesium sulfate and ammonium sulfate; *i.e.*, a *gradual change* in solubility with each increment of temperature.

The use of sodium sulfate for the precipitation of proteins is not a new procedure, nor is the use of it in the determination of the blood proteins a new process. Pinkus (2) realized the advantages of sodium sulfate in the study of proteins. He outlined the possibilities for its use and showed that (1) sodium sulfate possesses at 30°C. the same protein-precipitating power as ammonium sulfate. (2) When the anhydrous salt is used instead of the hydrated salt, at the temperature indicated, it precipitated globulins at the point of half saturation (about 25 per cent) and albumin at full saturation (about 50 per cent), it also allows of a fractionation of the proteins of Witte's peptone. (3) The use of sodium sulfate presents the following advantages: (*a*) the color reactions are hardly at all interfered with, (*b*) the nitrogen of the precipitate may be estimated directly according to method of Kjeldahl, (*c*) it is easy to obtain solutions containing little salt (5 per cent) by cooling, and (*d*) the salt itself is practically non-toxic. (4) By adding to protein solutions enough anhydrous sodium sulfate to absorb all of the water, a product is obtained which can be kept without change in the protein and is easily workable.

Porges and Spiro (3) confirmed the observation of Pinkus with regard to the precipitation limits of sodium sulfate: that the limits in terms of percentage of a saturated solution are approximately the same as those pertaining to ammonium sulfate. These authors used sodium sulfate in the quantitative separation of the serum proteins. They present evidence pointing toward the presence of three globulins, a euglobulin and two pseudoglobulins, with precipitation limits at 28 to 36 per cent, 33 to 42 per cent, and 40 to 46 per cent of saturated

sodium sulfate estimated from their published chart. Haslam (4) made use of sodium sulfate in the separation of albumoses.

Homer (5) has recently used sodium sulfate in the concentration of antitoxin and finds that antitoxin may be concentrated with sodium sulfate without denaturing by heat fully as well as with ammonium sulfate with heat. The removal of the salt is, moreover, simplified when sodium sulfate is used. Miss Homer constructed curves showing the percentage of protein precipitated by increasing amounts of ammonium sulfate and of sodium sulfate under various conditions of acidity and heat treatment and found them to be similar. She did not find critical points in the curves for the precipitation of eu- or pseudoglobulin or of serum albumin from undiluted serum. Mellanby (6) had previously failed to find critical zones with ammonium sulfate or magnesium sulfate. The following limits for the precipitation of the serum protein at 35–40°C. were found: pseudoglobulin precipitation complete at 11.5 to 18.5 per cent of anhydrous sodium sulfate, albumin completely precipitated at 32 per cent of anhydrous sodium sulfate. The precipitation of the individual proteins began at, and was complete at, lower concentrations of ammonium sulfate or of sodium sulfate than was required for their precipitation from their respective separate solutions in saline solution. There was also a greater overlapping of the precipitation limits for the individual proteins than was found for their separate solutions. The limits given hold for concentration of protein between 6.5 and 10 per cent. When the concentration of protein was reduced to 2.5 per cent, 34 gm. of sodium sulfate in 100 cc. were required for precipitation. Further dilutions required an increase in the concentration of the precipitating salt. The concentration of the electrolyte required for precipitation was affected by the reaction of the plasma, but a variation in reaction between pH 5.3 and 9.3 has only a slight effect on the result.

Spiro (7) has studied the effect of the addition of neutral salts upon the hydrogen ion concentration of protein solutions. The addition of either sodium sulfate or magnesium sulfate to a protein solution between the proportions of 1 part of salt to 9 parts of protein solution and 9 parts of salt and 1 of protein solution caused a change in the hydrogen ion concentration of the mixture from

pH 7.73 to 7.38 for sodium sulfate and pH 7.73 to 7.27 for magnesium sulfate. On the other hand, the addition of ammonium sulfate causes a change of hydrogen ion concentration of from pH 7.73 to 5.91.

In the light of the results obtained by Miss Homer and also Kauder (8) and Hofmeister (9) with regard to the variation in the quantity of salt required for the precipitation of proteins it became necessary to determine the optimum concentration of sodium sulfate for the precipitation of the total globulins. Dilution of serum has experimental evidence in its favor; Porges and Spiro and particularly Wiener (10) have diluted the serum before precipitation. Wiener, using ammonium sulfate, came to the conclusion that the accuracy of his results was enhanced by dilution; he obtained a smaller quantity of globulin under such conditions. Contrary to the findings of previous workers we found a critical zone and indications of more than one critical zone by using concentrations of sodium sulfate which differed from the preceding member of the series by 1 per cent of the anhydrous salt.

Determination of the Critical Zone for the Precipitation of Total Globulins.—A series of 50 cc. portions of sodium sulfate at 37°C. containing increasing quantities of sodium sulfate was measured into or prepared in wide mouth glass-stoppered bottles. 5 cc. of blood plasma were added to each bottle and the bottles placed in the incubator at 37°C. After 3 to 12 hours in the incubator the solutions were filtered and an aliquot, 25 cc., of the filtrate was taken for analysis. Nitrogen was determined by the method of Kjeldahl.[1] The results for the most complete experiments are given in Chart 1 together with other data concerning the range of constant nitrogen values.

Later, in our desire to make use of sodium sulfate as the precipitant of protein in the determination of globulin when using small quantities of plasma or serum, it seemed desirable to work at higher dilutions. In this work two procedures were adopted: (*a*) The plasma was diluted 1:10 with 0.8 per cent sodium chloride solution and 5 cc. of the diluted plasma, equivalent to 0.5 cc. of plasma, were added to 10

[1] The addition of potassium sulfate is not necessary since sodium sulfate can take the place of potassium sulfate in the digestion.

cc. of the required concentration of sodium sulfate; this gave a dilution of 1:30. (*b*) To 15 cc. of a given solution of sodium sulfate 0.5 cc. of plasma was added which gave a dilution of 1:31. The results obtained with these two procedures indicated that there was not an essential increase in accuracy by diluting the plasma before adding it to the salt solution. In later work, therefore, the salt solution was added directly to the serum.

Evidence showing the effect of dilution upon the precipitation of blood proteins was obtained from three sources: (*a*) by coagulating the proteins of different samples of plasma at increasing concentrations of sodium sulfate (Chart 1), these data served to establish the probable critical zone of precipitation; (*b*) by precipitating the same sample of plasma over a short series of increasing concentrations of sodium sulfate which covered the probable critical zone and in increasing dilution (see Table I); and (*c*) by using a short series of concentrations of sodium sulfate with a number of different plasmas. Data from the last set of experiments are not included in this paper but appear in part in the succeeding one since they are to be considered only as confirmatory results and are of particular value in connection with the relative accuracy of the macro method and the micro method described there.

Data are presented in Chart 1 which indicate that at 37°C. a zone exists between 20 and 22 per cent[2] of anhydrous sodium sulfate in which an increase of 1 per cent of sodium sulfate does not cause an increase in the quantity of protein precipitated which is greater than the error of the method (a maximum analytical error of approximately 1.5 per cent). On either side of this zone a change of 1 per cent in the concentration of the salt gives a result which is distinctly greater or less than the results obtained at the critical zone.

The critical zone between 20 and 22 per cent of anhydrous sodium sulfate was indicated by the results obtained in our first work at a dilution of 1:10.[3] These values do not hold absolutely for all degrees of dilution nor for all samples of blood as can be seen from Table I

[2] By 22 per cent of sodium sulfate we mean 22 gm. of sodium sulfate contained in 100 cc. of solution.

[3] As a convenience in measuring, 5 cc. of plasma were added to 50 cc. of salt solution which gave an actual dilution of 1:11.

and Chart 1. With increasing dilution of the plasma there is a tendency for the critical zone to shift in the direction of a higher concentration of salt, 21 to 23 per cent of sodium sulfate. Furthermore, at the higher dilutions the amount of protein precipitated is less than

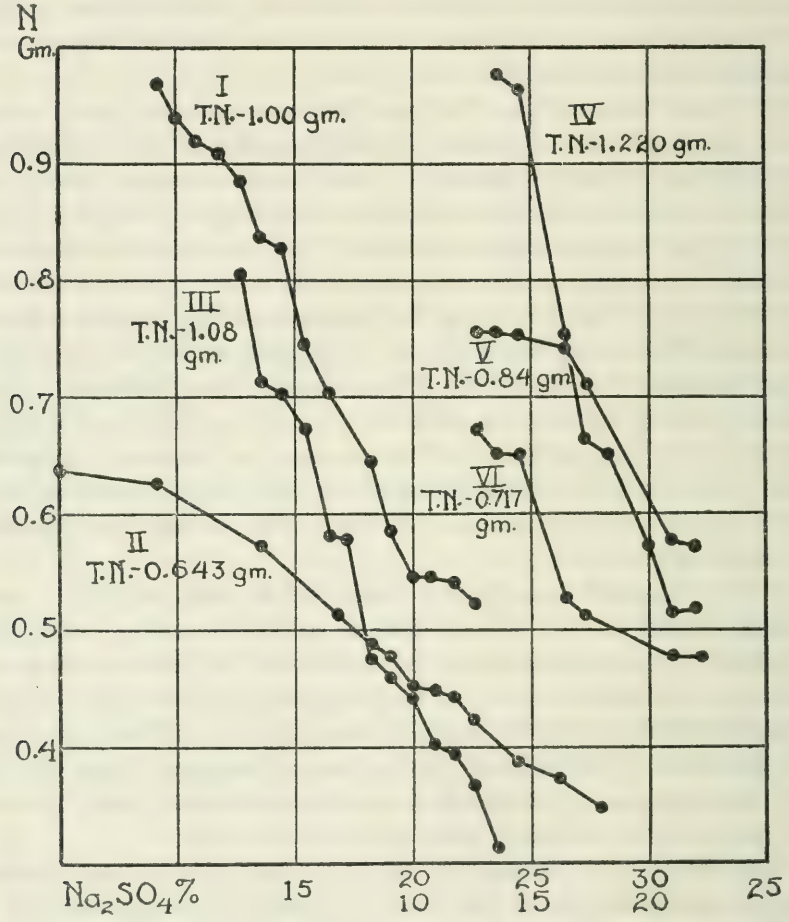

CHART 1. Curves showing the quantity of nitrogen, as grams of nitrogen per 100 cc. of serum or plasma, remaining in the filtrate after precipitation with increasing concentrations of sodium sulfate at 37°C. Curves I and II are for plasma and Curve III for serum at a dilution of 1:9.1. Curve IV is for plasma and Curves V and VI are for serum at a dilution of 1:32.3. The abscissæ are in terms of concentrations of anhydrous sodium sulfate expressed as per cent and the ordinates are expressed as grams of nitrogen in 100 cc. of blood.

at the lower dilutions. These facts have been noted previously for various salts by other investigators. The range of salt concentrations over which there is not a marked increase in precipitation for a small increment in salt may extend only over 2 per cent of sodium sulfate. On the other hand, a sharp break in the curve of precipitation with increasing salt concentration has always been observed at approximately the range of concentrations designated as the critical zone.

The variability of the critical zone with dilution and to a less extent with the sample of blood raises a question as to the value

TABLE I.

Data indicating the effect of the dilution of blood serum or plasma upon the precipitation of total globulins by sodium sulfate at 37°C. Results are expressed as grams of nitrogen per 100 cc. of plasma and represent the quantity of nitrogen remaining in the filtrate after precipitation.

Na_2SO_4	Dilutions.				
	1:1	1:10	1:20	1:30	1:40
per cent					
19	0.499	0.511	0.516	0.536	0.553
20	0.486	0.487	0.492	0.518	0.516
21	0.459	0.428	0.411	0.443	0.437
22	0.437	0.416	0.393	0.430	0.439
23	0.380	0.383	0.387	0.412	0.393
24	0.357	0.354	0.336	0.399	0.369
25	0.352	0.315	0.303	0.341	0.343

of the results and as to the concentration of sodium sulfate which will probably be in the critical zone if only one precipitation is adopted. Our observations have been in most cases on blood which is freshly drawn. In some cases determinations have been repeated on old plasma and have shown that in many cases 4 to 6 days later results obtained upon precipitation of the globulin are identical with those obtained with fresh blood. The fact is stated in support of the critical zone but not in support of the practice of analyzing old plasma. From the data presented and from that obtained in the analyses of blood in which two or more concentrations of sodium sulfate have been used, the precipitation of blood plasma or serum in a solution which

contains 21.5 gm. of anhydrous sodium sulfate per 100 cc. of solution, or 21.5 per cent, is most likely to correspond with the critical zone. At dilutions of plasma of 1:10 or higher the concentration given falls in the critical zone. The critical zone may be on either size of 21.5 per cent of the salt, but this concentration has been one of at least two concentrations in which the quantity of globulin precipitated is essentially equal. Ordinarily results obtained at 21 and 22 per cent of sodium sulfate fall in the critical zone.

Where it is desired to be assured of the presence of the critical zone determinations have been made at both 21 and 22 per cent of sodium sulfate. If both results at these concentrations agree it is assumed that the critical zone has been reached. If there is a marked difference in the results a precipitation is made at 23 per cent. In no case have we failed to obtain results in which one of the two pairs of concentrations showed an essentially constant degree of precipitation.

Ocular evidence has often been obtained of a change in the quantity of protein thrown down when the precipitation took place in test-tubes under which conditions the volume of precipitated protein shows a marked increase at the critical zone.

Comparisons of the protein precipitated by sodium sulfate at 21 to 22 per cent of sodium sulfate with that obtained with one-half saturated ammonium sulfate and magnesium sulfate at dilutions of 1:10 indicate that slightly less protein separates out when ammonium sulfate is used and more protein when magnesium sulfate is added. The results with magnesium sulfate have been repeatedly confirmed in other experiments. The data are contained in Table II.

Pinkus (2) gives 18.8 gm. of anhydrous sodium sulfate per 100 cc. as the point of complete precipitation of the globulin of ox serum $\frac{1}{4}$ dilute, horse serum $\frac{1}{15}$ dilute, serum globulin 7 per cent; and 20 gm. per 100 cc. solution for horse serum $\frac{1}{10}$ dilute and blood (ox) $\frac{1}{4}$ dilute. The results given above for serum diluted approximately 1:10 agree with that of Pinkus. On the basis of our results we feel justified in accepting the results obtained at 21.5 per cent of sodium sulfate as representing the precipitation of all globulins of blood serum or plasma.

The results of Porges and Spiro pointed to the possibility of other critical zones at approximately 12 to 14 per cent and at 18 per cent

of sodium sulfate. Working with increasing concentrations of sodium sulfate we found critical zones at approximately the above concentrations (see Chart 1). These zones correspond roughly with the point of beginning precipitation in the chart of Porges and Spiro. They are not, however, always as definite as the critical zone at 21.5 per cent of sodium sulfate. The zone at 12 to 14 per cent of sodium sulfate would correspond to euglobulin which is usually precipitated by dilution and acidification with carbon dioxide or acetic acid and sometimes with saturated sodium chloride. The zone at 17.4 per cent of sodium sulfate would correspond to the second globulin of Porges and Spiro which is sometimes designated pseudoglobulin I.

TABLE II.

Data relating to the quantity of nitrogen remaining in solution after precipitation with concentrations of sodium sulfate at the critical zone for total globulin and with one-half saturated ammonium sulfate and saturated magnesium sulfate at room temperature. Results are expressed as grams of nitrogen per 100 cc. of blood and represent the amount of nitrogen remaining in the filtrate after precipitation.

	Concentration.	Pig.	Goat.	Horse.
	per cent			
Sodium sulfate at 37°C.....	21.3	0.393	0.399	0.443
	22.5	0.402	0.383	0.430
	23.2	0.311	0.372	0.412
Ammonium sulfate.....		0.526	0.443	0.477
Magnesium "			0.323	0.385

Relation between the Quantity of Protein Precipitated at 13.4 Per Cent of Sodium Sulfate and with Sodium Chloride or Acidification with Carbon Dioxide.—It was assumed that the protein precipitated at the critical zone at about 13.4 per cent of sodium sulfate was probably euglobulin. If this was true the quantities of protein precipitated at this concentration and with other methods of precipitation should agree over a wide range of concentration of protein. Comparisons were, therefore, made of the quantity of protein precipitated by 13.5 per cent of sodium sulfate at 37°C., and by saturation of the diluted serum or plasma, with carbon dioxide, and with sodium

chloride at room temperature. For the carbon dioxide precipitations 5 or 0.5 cc. of serum or plasma were pipetted into a cylinder and 50 or 15 cc. respectively of distilled water added after which carbon dioxide was passed through the solution slowly for from ½ hour to 2 hours according to the procedure of Robertson (11). The solution was then filtered and aliquot portions of the filtrate were taken for analysis. For saturated sodium chloride similar quantities of serum or plasma were taken and 50 or 15 cc. of saturated sodium chloride added after which solid sodium chloride was added in excess. These solutions were permitted to stand for 12 hours with occasional shaking. Table III contains comparative results with the three methods.

TABLE III.

Results obtained by precipitation of different sera with (*a*) 13.5 per cent sodium sulfate, (*b*) saturated sodium chloride, and (*c*) saturation with carbon dioxide. Results are expressed as grams of nitrogen per 100 cc. of serum and represent the amount of nitrogen remaining in solution after precipitation.

Total nitrogen.	Sodium sulfate.	Sodium chloride.	Carbon dioxide.
0.658	0.595	0.621	0.573
0.665	0.665	0.661	0.578
0.693	0.600	0.569	0.621
0.695	0.613	0.615	0.652
0.696	0.630	0.615	0.652
0.731	0.595	0.661	0.600
0.863	0.718	0.792	0.735
0.901	0.665	0.718	0.569
1.046	0.915	0.929	0.915
1.255	1.000	0.997	0.957

The data cover a considerable range of protein concentrations and include some sera which contain practically no euglobulin. The average difference between the results obtained with sodium sulfate and with saturated sodium chloride is a precipitation of 0.027 gm. less of protein nitrogen per 100 cc. of blood with sodium chloride than with sodium sulfate. With relation to carbon dioxide, there was a precipitation of 0.021 gm. more protein nitrogen per 100 cc. of blood by carbon dioxide than with sodium sulfate. The average difference between results is not much beyond the experimental error

but the general trend of the results is characteristic of the procedure. Similar results have been obtained in a larger number of cases with sodium chloride and sodium sulfate which give an average difference, 0.018 gm. of protein nitrogen, which is approximately the same as the one given above. Comparisons of the quantity of protein precipitated at 12.5, 13.5, and 14.5 per cent of sodium sulfate have shown repeatedly that the amount of protein precipitated at 13.5 and 14.5 per cent of sodium sulfate is essentially the same and is always more than that precipitated by 12.5 per cent of sodium sulfate. The selection of 13.5 per cent of sodium sulfate at 37°C., we believe, as nearly represents the euglobulin fraction as can be determined by such quantitative procedures.

There is an advantage in using sodium sulfate instead of carbon dioxide or sodium chloride in that very constant conditions can be maintained. With carbon dioxide losses by evaporation and frothing are to be contended with, while with sodium chloride there is always the possibility that saturation is not complete.

The Presence of Pseudoglobulin, Precipitated at 17.4 Per Cent of Sodium Sulfate, in Blood.—The presence of two pseudoglobulins in blood has often been discussed. Porges and Spiro have presented evidence in favor of the occurrence of two pseuodoglobulins as the result of their precipitations from dilute serum with magnesium sulfate, sodium sulfate, ammonium sulfate, and other salts. Haslam (12) has definitely concluded that there are not more than two serum globulins in blood serum, euglobulin and pseudoglobulin. His statement is based upon results obtained after repeated precipitation of globulins with ammonium sulfate. The problem was, therefore, in such a state that it did not seem that our somewhat indefinite, but still suggestive, critical zone at about 16.4 to 17.4 per cent of sodium sulfate was significant. We are not now assured of the significance of the precipitation at 17.4 per cent of sodium sulfate. Additional evidence has been obtained, however, which points very strongly toward the presence of a protein or protein complex whose precipitation is complete at approximately 17.4 per cent of sodium sulfate.

The evidence rests upon two facts, in addition to that already in the literature, (*a*) the indication of a critical zone at 16.4 to 17.4 per cent of sodium sulfate, already referred to, and (*b*) the *absence*

of protein which is precipitated at 16.4 to 17.4 per cent of sodium sulfate in certain blood. A precipitate first occurs at 18.4 per cent of sodium sulfate. Under suitable conditions the blood of the same animals will contain, within a few hours, large quantities of protein precipitable at 13.5 per cent of sodium sulfate, or by carbon dioxide, or saturated sodium chloride, and at 17.4 per cent of sodium sulfate. Other work associated with this and confirming this observation will be presented in due time.

The presence of a protein in this case rests upon the acceptance of the definition of a euglobulin as one which is precipitated by acidification with carbon dioxide in dilute solution. That the amount of protein precipitated by carbon dioxide from blood is practically the same as that obtained under two other conditions has just been discussed and points to the separation of at least a mixture of fairly definite composition. This being so the failure to obtain a precipitate until at least 18.4 per cent of sodium sulfate has been added to blood serum under some conditions and the presence of a precipitate under others is an indication of the existence of a protein or protein complex precipitable between the limits of 13.5 and 17.4 per cent of sodium sulfate.

The quantity of protein precipitated between 13.5 and 17.4 per cent of sodium sulfate in one series of experiments is roughly equal to that precipitated at 13.5 per cent. There are bloods in which the quantity of protein precipitated between these limits is greater than at 13.5 per cent of sodium sulfate. Judging from the volume of precipitate the greatest proportion of the precipitation may occur between 16.4 and 17.4 per cent of sodium sulfate. This fact made us hestitate between 16.4 and 17.4 per cent of sodium sulfate as the percentage which would represent the approximate completion of the precipitation of pseudoglobulin I. On the other hand, there are cases in which no precipitate occurs at 17.4 per cent of sodium sulfate. When the critical zone is most marked at the range of concentrations under consideration, 17.4 per cent of sodium sulfate is always one of the two concentrations involved and we have, therefore, adopted this concentration as the point which we consider as best representing the quantity of euglobulin plus pseudoglobulin I present in blood.

In case plasma is being studied the value at 17.4 per cent will represent fibrinogen, euglobulin, and pseudoglobulin I.

DISCUSSION.

Data have been presented which indicate that as increasing quantities of sodium sulfate are added to diluted serum or plasma or serum at 37°C. the amount of protein thrown out of solution increases. There are at least three points in such a series at which an increase of 1 per cent of sodium sulfate does not produce as large an increase in precipitation as will be caused by the concentration of salt preceding or succeeding these concentrations. These points, or critical zones, are at 13.5 to 14.5, 16.4 to 17.4, and 21 to 22 per cent of sodium sulfate. This is particularly true of the concentrations 13.5 to 14.5 and 21 to 22 per cent of sodium sulfate. At 16.4 to 17.4 per cent of sodium sulfate it is not always possible to demonstrate a critical zone.

Basing our conclusions on the quantity of protein precipitated as determined by analysis of the filtrate from such precipitations[4] two of these critical zones agree very closely with other methods for determining proteins. Results at the zone at 13.5 to 14.5 per cent of sodium sulfate agree closely with those obtained with saturated sodium chloride and carbon dioxide. At 21 to 22 per cent of sodium sulfate the results are approximately those obtained with magnesium sulfate and ammonium sulfate both of which have long been accepted as a means of completely precipitating globulins. For the acceptance of the zone at 17.4 per cent of sodium sulfate the evidence rests in part upon the absence of protein precipitable up to that concentration in certain bloods.

Whether or not results obtained by precipitation of proteins from a mixture of proteins with salts represent separations of pure proteins

[4] In using the procedures described it is assumed that the aliquot taken from the filtrate after precipitation contains a true proportionate amount of the unprecipitated protein and that there has not been any adsorption of the unprecipitated protein by the precipitated protein nor by the filter paper. The results of Spiro (13) indicate that precipitation is probably not complete and that there is a small amount of protein remaining unprecipitated. We have found that when diluted serum is filtered that there is a small loss of nitrogen in the process which may be slightly greater than the experimental error.

is an open question. The considerable mass of literature on this subject is in favor of the opinion that the protein thrown down is a mixture of proteins; (*a*) present as compounds, (*b*) due to the adsorption of other proteins by the precipitated protein, or (*c*) because the precipitation limits overlap. One fact stands out in our experiments, however, which is applicable to any concentration of salt; under constant conditions of temperature and concentration of salt a constant amount of protein is precipitated. Robertson (11) in developing his refractometric procedure for the determination of globulins substantiated his use of ammonium sulfate for the precipitation of total globulins upon the constancy of the results obtained with definite concentrations of salt. From our subsequent work with a method developed from our findings presented here we agree with Robertson in referring to precipitation with ammonium sulfate, that "if the proportion of this substance is different in the serum of different individuals or species, we may be fairly confident, therefore, that the quantitative relations of the globulin and albumin," or intermediate globulin, "groups are different in these animals."

Our work has covered a number of species of mammals having blood of varying protein content and the limits given have held in every case. On the other hand, in carrying out a problem involving the determination of serum or plasma proteins we would strongly advise the use of two succeeding concentrations of salt at the critical zone until it had been determined that the critical zone was present at the concentrations of salt used in that particular case.

SUMMARY.

1. Sodium sulfate solutions at 37°C. may be used to precipitate the proteins of blood into fractions corresponding to those usually separated by carbon dioxide or saturated sodium chloride, euglobulin, and magnesium sulfate or ammonium sulfate, total globulin.

2. Critical zones in the curve representing the precipitation of protein with increasing salt concentration have been located at 13.5 to 14.5, 17.4, and 21 to 22 per cent of anhydrous sodium sulfate at 37°C. For the purpose of estimating the quantity of protein present at these zones, 13.5, 17.4, and 21.5 per cent of sodium sulfate is recommended.

3. Evidence has been presented which tends to substantiate the presence of two globulins in blood serum in addition to euglobulin; pseudoglobulin I and pseudoglobulin II whose precipitations are complete at approximately 17.4 and 21 to 22 per cent of sodium sulphate respectively.

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THE DETERMINATION OF PROTEINS IN BLOOD—A MICRO METHOD.

By PAUL E. HOWE.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

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The use of sodium sulfate instead of ammonium sulfate in the precipitation of globulins makes it possible to determine the proteins of plasma or serum in small quantities of blood. The basis for the selection of sodium sulfate has been discussed in the preceding paper (1). Robertson (2) described a procedure for the determination of blood proteins in small quantities of blood with the aid of the refractometer. Cullen and Van Slyke (3) have proposed a procedure which gives consistent results and which does not require any special apparatus beyond that to be found in any laboratory. Their method, however, required 5 cc. of plasma for each constituent of the blood determined. The determinations described below can be performed with the usual laboratory apparatus and require but 0.5 cc. of plasma or serum for each determination.

The procedures involve the precipitation of fibrinogen with calcium chloride, the globulins with definite concentrations of sodium sulfate at 37°C., and non-protein nitrogen with trichloroacetic acid. In the case of fibrinogen and non-protein nitrogen the technique of Cullen and Van Slyke is followed. The globulins are precipitated by adding a concentration of sodium sulfate which is greater than the required percentage by the amount of sodium sulfate necessary to produce the desired percentage when added to the blood sample. The solutions are prepared by dissolving the required quantity of sodium sulfate in a little less than the final volume, which requires heat for the higher percentages, and then diluting to volume at 37°C. All precipitations and filtrations with sodium sulfate are carried out in the incubator or hot room. The following concentrations of sodium sulfate are needed: 14, 18, and 22.2 per cent. When 15 cc. portions

of these solutions are added to 0.5 cc. of blood the final concentrations are approximately 13.5, 17.4, and 21.5 per cent of sodium sulfate respectively. At 13.5 per cent of sodium sulfate euglobulin is precipitated, at 17.4 per cent euglobulin and pseudoglobulin I are precipitated, and at 21.5 per cent all globulins are thrown out of solution. In case blood plasma is used fibrinogen is present in each case and the nitrogen representing this protein must be deducted. Suitable blanks must be made for each determination.

The volume of solution used, 15.5 cc. (15 cc. of salt solution plus 0.5 cc. of blood), permits duplicate determinations to be made on each precipitation. This does not insure against errors in precipitation, but it has been our experience that simultaneous duplicate precipitations almost invariably agree. When it is desired to make duplicate precipitations it is advised that 13.5 and 14.5 per cent, 16.4 and 17.4 per cent, and 21 and 22 per cent of sodium sulfate, final concentrations, be used. With these concentrations the values obtained with each pair should agree within experimental error, except perhaps in the case of 16.4 and 17.4 per cent of sodium sulfate.

Precipitations are made in test-tubes or 50 cc. centrifuge tubes and then closed with a rubber stopper. The filtrations are conducted in the incubator using a dry 9 cm. filter paper. It is desirable to wet the filter paper with a small amount of the solution to be filtered before pouring on the bulk of the solution containing the precipitate. The funnels are covered with watch-glasses. 1 inch test-tubes held in test-tube racks are convenient for filtration. With these tubes and the cover-glasses a reasonably tight filtration system is obtained.

For measuring, the accurately calibrated Ostwald pipettes and the 15 cc. graduated pipettes introduced by Folin are used.

The nitrogen determinations are conducted in large Pyrex test-tubes in general according to the original micro procedure of Folin and Farmer (4), and the distillations are carried out, according to the procedure of Folin and Wu (5) in their system of blood analysis, without cooling the distillate. In distilling, a Pyrex connecting tube is used which carries a distilling head and has an enlargement on the tube which dips into the acid to guard against mechanical transfer of alkali and back suction. For titrations we use standard hydrochloric acid and sodium hydroxide which are approximately 0.05 and

0.025 N respectively. Our burettes deliver 25 cc. and are graduated to 0.05 cc. Methyl red is used as an indicator.

The determinations are as follows: Plasma is collected so that it contains 0.5 per cent of potassium oxalate. Both plasma and serum are centrifuged until clear.

Total Nitrogen.—0.5 cc. of plasma or serum is placed in a large Pyrex test-tube and the 2 cc. of concentrated sulfuric acid, 1 drop of 5 per cent copper sulfate, and a quartz pebble are added; the solution is digested over a free flame until clear, and then 7 to 10 minutes longer. Cool 3 to 5 minutes, add 25 to 30 cc. of ammonia-free distilled water, a small amount of talcum powder or powdered pumice stone, and concentrated sodium hydroxide solution sufficient to neutralize the acid, and distil into standard acid.

In place of using two 0.5 cc. portions of blood 15 cc. of 0.8 per cent sodium chloride solution may be added to one portion of 0.5 cc. and two 5 cc. portions of the diluted plasma taken for analysis.

Fibrinogen.—0.5 cc. of plasma is measured into a tube, 14 cc. of 0.8 per cent sodium chloride solution at room temperature are added, then 1 cc. of 2.5 per cent calcium chloride, a small crystal of thymol, and the tube is stoppered. The tube and contents are allowed to stand until the fibrin is formed and then filtered on a dry filter. Two 5 cc. portions of the filtrate are taken for analysis.

Euglobulin.—0.5 cc. of plasma or serum is measured into a tube, 15 cc. of 14 per cent anhydrous sodium sulfate at 37°C. and a little thymol are added, and the tube is stoppered, shaken, and allowed to stand for at least 3 hours, or until the precipitate has settled. The solution is then filtered through a dry filter and two 5 cc. portions are taken for analysis. The results represent euglobulin in the case of serum and fibrinogen plus euglobulin in the case of plasma.

Euglobulin Plus Pseudoglobulin I.—The procedure is the same as for euglobulin except that 18 per cent sodium sulfate is used.

Total Globulins.—The procedure is the same as in euglobulin except that 22.2 per cent of sodium sulfate is used.

Non-Protein Nitrogen.—0.5 cc. of plasma or serum is measured into a tube and 15 cc. of 5 per cent trichloroacetic acid at room temperature are added. The remainder of the procedure is the same as in euglobulin.

The calculations of nitrogen are those ordinarily associated with Kjeldahl determinations. The volume of solution from which the aliquot portions for analysis are taken is 15.5 cc. We have expressed our results in terms of grams of nitrogen in 100 cc. of blood. As the result of the analytical procedures the following results can be calculated for serum:

Total nitrogen.

Euglobulin nitrogen = Total nitrogen – nitrogen in filtrate from 13.5 per cent sodium sulfate precipitation.

TABLE I.

Comparative results obtained with the method of Cullen and Van Slyke "macro" and the micro method. Results are expressed as grams of nitrogen per 100 cc. of blood remaining in the filtrate after precipitation.

	Cow.		Pig.		Goat.		Horse.	
	Macro.	Micro.	Macro.	Micro.	Macro.	Micro.	Macro.	Micro.
Total N		1.67	1.05	1.05		1.10		1.08
$CaCl_2$	1.31	1.39	0.91	0.92	0.98	0.97	1.03	0.97
Saturated NaCl	1.01	1.00	0.80	0.81	0.84	0.83	0.90	0.89
Sodium sulfate.								
13.5 per cent		1.01		0.74		0.84		0.88
20.9 " "	0.31		0.41		0.39		0.43	
21.3 " "		0.29		0.39		0.40		0.44
22.5 " "		0.27		0.40		0.38		0.43
23.2 " "		0.27		0.31		0.37		0.41
Trichloroacetic acid	0.06	0.07	0.03	0.02	0.05	0.07	0.04	0.06
Ammonium sulfate			0.53		0.44		0.48	
Magnesium "					0.32		0.39	

Pseudoglobulin I nitrogen = Nitrogen in filtrate from 13.5 per cent sodium sulfate precipitation – nitrogen from 17.4 per cent sodium sulfate precipitation.

Pseudoglobulin II nitrogen = Nitrogen in filtrate from 17.4 per cent sodium sulfate precipitation – nitrogen in filtrate from 21.5 per cent sodium sulfate precipitation.

Total globulin nitrogen = Total nitrogen – nitrogen in filtrate from 21.5 per cent sodium sulfate precipitation.

Albumin nitrogen = Nitrogen in filtrate from 21.5 per cent precipitation – non-protein nitrogen.

Non-protein nitrogen = Nitrogen in filtrate from trichloroacetic acid precipitation.

For plasma the euglobulin is estimated by substracting the filtrate nitrogen from the nitrogen in the filtrate after the precipitation of fibrinogen.

Table I contains some comparative data on the Cullen-Van Slyke procedure and the micro method. Determinations using sodium sulfate and magnesium sulfate in the Cullen-Van Slyke method are included. Determinations for pseudoglobulin I are not included in the table since they were not made at the time the plasma was analyzed. The results are expressed in terms of the nitrogen remaining in the filtrate after precipitation without calculating the various fractions.

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AN EFFECT OF THE INGESTION OF COLOSTRUM UPON THE COMPOSITION OF THE BLOOD OF NEW-BORN CALVES.

By PAUL E. HOWE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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An interesting relation between the ingestion of colostrum and the composition of the blood of new-born calves was observed in the course of a study in progress in this department. It was found that the blood serum of a new-born calf before it has nursed does not contain proteins precipitable by 17.4 per cent anhydrous sodium sulfate, or at least but a trace of precipitation occurs at this concentration. Precipitates do not occur with concentrations less than 17.4 per cent of sodium sulfate, with saturated sodium chloride, nor with carbon dioxide. If we accept the usual means of separating the proteins of blood the failure to obtain a precipitate under the above conditions amounts to saying that the blood of a new-born calf *does not contain euglobulin nor pseudoglobulin I.* After the calf has received colostrum the blood serum contains relatively large amounts of protein precipitable by carbon dioxide, saturated sodium chloride, and 13.5 per cent sodium sulfate—euglobulin—and also at 17.5 per cent of sodium sulfate—pseudoglobulin I.[1] On the other hand, if the calf is given ordinary whole milk from a cow well along in lactation, or receives milk from a cow which has not been "dried off" before parturition, *i.e.* has been milked up to or close to the time of parturition, conditions in which very little globulin is ingested, the quantity of euglobulin or pseudoglobulin in the blood is neglible. The subsequent ingestion of colostrum after a period, so far observed,

[1] These proteins may be absent from the blood of older calves. The conditions under which this occurs have not been, as yet, determined. So far as we have examined the blood of adult animals, the serum always contains euglobulin and pseudoglobulin I.

of 21 hours results in the appearance of the globulins in the blood. The above facts relate to blood serum. Fibrinogen is present in

TABLE I.

Data Showing the Relation between the Ingestion of Colostrum and the Composition of the Blood of New-Born Calves. Results are Expressed as Gm. of Nitrogen in 100 Cc. of Blood.

Remarks.	Total nitrogen.	Euglobulin.	Pseudo-globulin I.	Pseudo-globulin II.	Total globulin.	Albumin.	Non-protein nitrogen.
Calf A.							
5 hrs. after birth. No colostrum	0.596	0.023	0.004	0.140	0.167	0.376	0.053
6 hrs. after receiving colostrum. Age 11 hrs.	0.838	0.173	0.235	0.087	0.505	0.280	0.053
1 day old	1.028	0.337	0.217	0.131	0.765	0.210	0.053
2 days "	0.962	0.271	0.310	0.075	0.656	0.253	0.053
Calf B.							
5 hrs. after birth. No colostrum	0.605	0.000	0.009	0.140	0.141	0.390	0.074
16 hrs. after receiving whole milk. Age 21 hrs.	0.633	0.000	0.040	0.135	0.173	0.386	0.074
5 hrs. after receiving colostrum from mother. Age 26 hrs.	0.632	0.000	0.074	0.140	0.216	0.363	0.053
17 hrs. after receiving colostrum. Age 43 hrs.	0.668	0.032	0.084	0.101	0.222	0.393	0.053
4 days old	0.687	0.048	0.144	0.110	0.312	0.332	0.053
Calf C.							
At birth	0.595	0.000	0.096*	0.101	0.197	0.350	0.048
6 hrs. after birth. No milk	0.625	0.000	0.097*	0.083	0.157	0.415	0.053
17 hrs. after nursing. Mother not "dried off"	0.647	0.039		0.192	0.231	0.363	0.053
24 hrs. after nursing mother. Age 47 hrs.	0.668	0.003	0.168	0.022	0.183	0.332	0.053
2 days old	0.643	0.032	0.153	0.035	0.223	0.367	0.053
3 " "	0.611	0.051	0.149	0.035	0.235	0.323	0.053

* In these cases there was practically no protein precipitated at 16.4 per cent of sodium sulfate. The results given are based on data obtained at 17.4 per cent of sodium sulfate.

the blood of a new-born calf and is precipitated under all of the conditions mentioned above.

In the study of the changes which take place in the blood of young

calves the recently published method[2] was used, which involves the use of sodium sulfate at 37°C. The data of Table I serve to illustrate the changes which take place in the composition of the blood of the calf during the early days of its life.[3]

Calf A received colostrum within 5 hours after birth. Calf B was fed whole milk 5 hours after birth and then its mother's colostrum which had been held in the refrigerator for 16 hours. The mother of Calf C was milked up to the time of parturition.

The absence of euglobulin and pseudoglobulin I from the blood of new-born calves and its sudden appearance after ingesting colostrum brings the relation of colostrum to the new-born into a new light. It would appear that the calf receives its first supply of these globulins from the colostrum and that if they are not acquired from the colostrum it probably takes some time to form them. We are now studying the conditions under which these globulins are produced.

It has repeatedly been shown that colostrum is rich in globulins and that later the quantity of globulin in milk is practically negligible; the presence of globulin has, in fact, often been questioned. The function of colostrum has not been well understood; the most common explanation is that it acts as a purgative. It has been observed that an animal can live and grow without colostrum. Hohlfeld[4] found that dogs, goats, and guinea pigs could be raised without receiving colostrum but that better growth in early life occurred when colostrum was taken. He ascribed the value of colostrum to its higher nutritive value, which enabled the young with a small stomach capacity to obtain sufficient nourishment in a relatively small volume. We have observed the effect of the absence of colostrum upon the fat

[2] Howe, P. E., *J. Biol. Chem.*, 1921, xlix, 109.

[3] There is some difference of opinion with regard to the significance of results obtained by precipitation of proteins from a mixture of proteins, such as blood serum, due to the overlapping of the precipitation limits or the adsorption of unprecipitated protein by the precipitated protein. The data in Table I indicate that for comparative studies the results have some significance. The wide range in variation in the amount of globulins does not appear to have the marked effect upon the various fractions estimated which one would expect if the interfering factors played an important rôle in the processes involved.

[4] Hohlfeld, M., *Arch. Kinderheilk.*, 1907, xlvi, 161.

content of the feces of young calves[5] and found that the appearance of an increased fat content which occurs normally in about 3 days was delayed but that it appeared subsequently.

It is a pleasure to record our thanks to Dr. R. B. Little for the opportunity of analyzing blood serum collected by him in the course of his experiments.

[5] Howe, P. E., *Am. J. Dis. Child.*, 1921, xxi, 57.

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